THE PITUITARY GONADOTROPIN-RELEASING HORMONE (GNRH) RECEPTOR OF THE FEMALE RABBIT: CHARACTERIZATION AND DEVELOPMENTAL ASPECTS

by

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ABSTRACT

The aim of the present study was to characterize the pituitary GnRH binding site in the rabbit and investigate its possible role in sexual maturation of the female rabbit. A radioligand binding assay was established and the presence of specific ^{125}I-DAla^{6}EA binding sites in the anterior pituitary gland of the rabbit was demonstrated. ^{125}I-DAla^{6}EA binding was saturable, specific, displaceable, reversible, correlated with increasing tissue concentrations and susceptible to physiological manipulation. Significant ^{125}I-DAla^{6}EA binding was not present in the rabbit ovary suggesting that GnRH or GnRH-related peptides are not directly involved in the control of luteal function of the rabbit. ^{125}I-DAla^{6}EA binding indicated the presence of two binding sites in the female adult rabbit pituitary; a high affinity, low capacity site (K_d = 0.3-0.4 nM; B_{max} = 100-200 fmol/mg protein) and a lower affinity, high capacity site (K_d = 30 nM; B_{max} = 5-8000 fmol/mg protein). Ontogeny of ^{125}I-DAla^{6}EA binding in the female rabbit (40 to 120 days of age) did not show a correlation between binding site number and serum LH. In addition, the net serum LH response in female rabbits to a subcutaneous injection of DAla^{6}EA (10ng, 100ng, 1μg per kg body weight) was not significantly different between animals 40, 75 and 120 days of age. This suggests that a decrease in pituitary responsiveness to GnRH is not associated with sexual maturation in the female rabbit. Results indicate that factors other than and/or in addition to GnRH binding site number, such as post-receptor events play a role in gonadotropin secretion in the female rabbit.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ANOVA	analysis of variance
AVP	arginine vasopressin
[B]	concentration of bound hormone
B_{Eq}	amount of hormone bound at equilibrium
BSA	bovine serum albumin
B_{max}	maximal binding capacity
B_t	amount of bound ligand at time t
°C	degrees Celsius
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CaM	calmodulin
cAMP	cyclic adenosine 3',5' monophosphate
DAla^{6}EA	des-Gly^{10},[DAla^6]GnRH ethylamide
ddH_20	double distilled water
dH_20	distilled water
DG	1,2-diacylglycerol
DLeu^{6}EA	des-Gly^{10},[DLeu^6]GnRH ethylamide
DpGlu^1	[D-pGlu^1,D-Phe^2,D-Trp^{3,6}]GnRH antagonist
DTT	dithiothreitol
ED_{50}	effective dose giving half-maximal response
E_q	equilibrium
E_2	estradiol
ETOH	ethyl alcohol
[F]	concentration of free hormone
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
[H]	concentration of free hormone
HCl	hydrochloric acid
H & E	hematoxylin and eosin
[HR]	concentration of hormone and receptor complex
H_t	total amount of free hormone
^{125}I	radioactive iodine isotope
I	inositol
IC_{50}	concentration of competing ligand which causes 50% inhibition of radioligand binding
IP	inositol 1-phosphate
IP_2	inositol 1,4-bisphosphate

IP_3	inositol 1,4,5-trisphosphate
K_a	apparent association constant
K_d	apparent dissociation constant
k_1	association rate constant
k_2	dissociation rate constant
[L]	concentration of ligand
LH	luteinizing hormone
Li^+	lithium
mCi	milli Curie
MG	monoacylglycerol
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
NSB	non specific binding
NZW	New Zealand White
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEI	polyethylenimine
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
$[^{32}]P_i$	[^{32}P]orthophosphate
PI	phosphatidylinositol/phosphoinositide
PL	phospholipid
PIP	phosphatidylinositol-4-phosphate
PIP_2	phosphatidylinositol-4,5-bisphosphate
PLC	phospholipase C
PO_4	phosphate
PKC	protein kinase C
PS	phosphatidylserine
[R]	concentration of free receptor
RIA	radioimmunoassay
SA	specific activity
sc	subcutaneous
SD	standard deviation
SEM	standard error of the mean
Tris	tris(hydroxymethyl) aminomethane
TSH	thyroid stimulating hormone

this thesis is dedicated to P.V.D

CHAPTER ONE

INTRODUCTION

1.1 RATIONALE

Sexual maturation in mammals is characterized by various changes in the hypothalamic-pituitary axis (for reviews see Odell, 1990; Grumbach and Kaplan, 1990). The processes involved in initiating sexual maturation are complex and incompletely understood. Hypothalamic content of gonadotropin releasing hormone (GnRH;LHRH) (Aubert et al, 1985; Ojeda et al, 1976; Araki et al, 1975) and pituitary content of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) have been shown to increase with age in the female rat (Dohler et al, 1977; Kragt and Ganong, 1968). Changing patterns of serum gonadotropins and gonadal steroids have been documented during sexual maturation (Ojeda and Urbanski, 1988; Dalkin et al, 1981). In the prepubertal female rat, plasma FSH and LH reach a peak at 15 days of age and decline by 25 days (Chan et al, 1981). Similarly, pituitary responsiveness to exogenous GnRH increases with age and is maximal at 10-25 days in the female rat followed thereafter by a decline prior to first ovulation (Ojeda et al, 1977; Debeljuk et al, 1972). Peak responses to GnRH in the rat have been correlated with maximal GnRH binding suggesting a mechanism by which pituitary responsiveness is altered during sexual maturation (Duncan et al, 1983; Dalkin et al, 1981).

Recently, an age-related prepubertal increase in gonadotropin secretion has been demonstrated in the female rabbit (YoungLai, 1986; deTurckheim et al, 1983). Discrete peaks in concentrations of LH and FSH were observed at 3-9 days and 30-

50 days after birth (YoungLai, 1986; Wilkinson and YoungLai, 1986). Puberty normally occurs at approximately 100 days (YoungLai, 1986; deTurckheim et al, 1983). Nevertheless, the exact mechanism involved in sexual maturation in the female rabbit remains uncertain. The gonadotropin pattern probably reflects a complex interaction between gonadotropin synthesis and release in response to GnRH and modulation by circulating steroids. One possible locus for changes associated with sexual maturation is the interaction between GnRH and its pituitary binding site. Changes in pituitary GnRH binding could occur from alterations in binding affinity and/or binding concentration. Thus, the investigation of the pituitary GnRH binding site in the female rabbit during sexual development may further our understanding of the complex mechanism of sexual maturation.

1.2 HYPOTHESIS

Sexual maturation in the female rabbit is characterized by a decrease in the number of pituitary GnRH binding sites.

1.3 SPECIFIC AIMS

(1) To fulfil the criteria for receptor validation: characterization of the pituitary GnRH binding site using the radioligand binding assay.

(2) To quantitate pituitary GnRH binding at various ages and relate the fundamental characteristics of binding sites (K_d, B_{max}) to the known pattern of

gonadotropin secretion (YoungLai, 1986) and hence sexual maturation.

(3) To ascertain possible changes in pituitary responsiveness during sexual maturation of the female rabbit.

(4) To identify the presence or absence of GnRH binding sites in the rabbit ovary.

1.4 ANIMAL MODEL

The rat as an animal model for the study of sexual maturation is very convenient and has been extensively used (Ojeda and Urbanski, 1988). However, a striking difference exists between the rat and the human, namely, the absence in the rat of the juvenile hiatus of gonadotropin secretion that is characteristic of the primate (Plant, 1988). In contrast to the rat, recent evidence in the female rabbit reports increases in LH and FSH secretion at approximately 3-9 and 30-50 days of age followed by low gonadotropin levels (YoungLai, 1986). Sexual maturation, as indicated by sexual receptivity, mating and ovulation, normally occurs at approximately 100 days (YoungLai, 1986; deTurckheim et al, 1983). Thus, the rabbit provides a more suitable model to investigate the variables associated with pubertal changes as noted in humans. In addition, the immature rabbit, because of its size, permits multiple blood samples to be obtained readily which allows for a detailed analysis of the hormonal changes responsible for possible mechanisms involved in sexual maturation.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 INTRODUCTION

Gonadotropin releasing hormone (GnRH) is a decapeptide which is synthesized in specialized neurosecretory cells of the medial-basal hypothalamus. The recognition and characterization of GnRH was largely due to the Nobel prize work of Drs. Guillemin and Schally in the early 1970s (Burgus et al, 1972; Schally et al, 1971). Its amino acid sequence is:

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Burgus et al, 1972).

GnRH is released in a pulsatile pattern into the hypothalamo-hypophyseal portal circulation and travels to the anterior pituitary to stimulate the secretion of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). The first step in the mechanism by which GnRH evokes gonadotropin release is the binding of GnRH to specific receptors located on the surface of gonadotrope cells. The present chapter will review the following aspects of GnRH and GnRH receptor binding: (a) general properties of receptors (b) the GnRH receptor (c) the GnRH effector mechanism and (d) regulation of the GnRH receptor.

Please note that the term 'receptor' will be used although it is recognized that the term 'binding site' may be more appropriate in some cases.

2.2 RECEPTORS

The initial step in the action of a hormone is its interaction with a specific binding protein known as a receptor, located in or on the target cell (Bolander,

1989). The concept of receptors was first proposed by Langley over 100 years ago (Langley, 1878). He noted that curare could block the effects of nerve stimulation in muscle contraction yet direct muscle stimulation was unaffected. Langley concluded that curare must be acting on what he termed a "receptive substance" that was responsible for triggering muscle contraction (Langley, 1905). Thus, the function of a receptor is to recognize a specific ligand and then convert its information into a signal that results in a physiological response (Hollenberg, 1985; Laduron, 1984). This dual function of receptors (recognition and activation) distinguishes receptors from other molecules such as acceptors (Hollenberg, 1985).

2.2.1 TYPES OF RECEPTORS

There are three basic types of hormone receptors which are classified according to their cellular location (Bolander, 1989). Peptides and other hydrophobic hormones (eg. catecholamines) have receptors that are integral membrane proteins and whose extracellular domain contains the binding activity (Bolander, 1989). Steroid hormone receptors were initially believed to exist in the cytoplasm or nucleus of the cell but recent investigations suggest that most steroid hormone receptors are exclusively nuclear (Carson-Jurica et al, 1990). Thyroid hormone receptors are located on the nuclear chromatin (Oppenheimer et al, 1987). Regardless of the type, binding of the ligand to its receptor site is highly specific, and in the case of an agonist, will subsequently alter the functioning of the target cell. The remainder of

this chapter focuses on cell surface receptors, in particular the GnRH receptor.

2.2.2 HORMONE-RECEPTOR INTERACTIONS

Binding assays that study hormone-receptor interactions use the Law of Mass Action to describe the binding of the hormone to the receptor. The simplest model of hormone-receptor action can be described at equilibrium (E_q) as follows:

$$[H] + [R] \underset{k_1}{\overset{k_2}{\rightleftharpoons}} [HR] \qquad (1)$$

where [R] is the concentration of free receptor; [H] is the concentration of free drug or hormone; [HR] is the concentration of hormone-receptor complex; k_1 is the association rate constant and k_2 is the dissociation rate constant. The equilibrium or apparent dissociation constant (K_d) which is used as an estimate of the affinity of the ligand for the receptor is represented by k_2/k_1:

$$K_d = [H][R]/[HR] = k_2/k_1 \qquad (2)$$

This simple equation forms the basis of models of drug action such as the occupancy theory (Clark, 1926; Gaddum, 1926) and the rate theory (Paton, 1961). However, the equation assumes that the binding site (a) does not interact with other binding sites and (b) does not change binding characteristics once occupied by the ligand (Hollenberg, 1985).

It is now recognized that a simple model is not adequate to describe receptor-hormone interactions. The receptor may exist in a variety of conformational states

subsequent to ligand binding. A theoretical model to describe the sequence of events is:

$$H+R \rightleftharpoons HR' \rightleftharpoons HR^* \rightleftharpoons H+R^* \rightleftharpoons R'' \rightleftharpoons R \qquad (3)$$

(Hollenberg, 1985)

In this model H represents the hormone and R the receptor in the non-activated state; R' represents the receptor in the conformationally altered active state; the inactive receptor during and after occupancy are represented by HR^* and R^*, respectively; R" is no longer capable of binding the hormone (*i.e.* totally inactive). A further event may convert the R" inactivated form into R which is capable of binding the hormone (Hollenberg, 1985). However, the theoretical possibility of a receptor converting into a form which cannot bind any ligand during a state of equilibrium has recently been questioned (Ehlert, 1988).

A modification of the traditional occupancy model of hormone-receptor interaction resulted when it was found that different agonists, acting at the same receptor, produced different maximal effects in the same tissue. This observation led to the concept of 'full' or 'partial' agonists. Two aspects of receptor-hormone interaction had to be considered: (a) recognition of the hormone by the receptor and (b) the ability of the hormone-receptor complex to elicit a biological response. The terms 'intrinsic activity' (Ariens, 1954) and 'efficacy' (Stephenson, 1956) were introduced to describe this response-eliciting power of the agonist. The effect (E) of a drug could be defined as $E=ey$ (where e represents the efficacy and y is the

fraction of receptors occupied by the agonist)(Stephenson, 1956). According to this theory, efficacies for antagonists and full agonists would be zero and unity, respectively, while partial agonists would obtain intermediate values (Hollenberg, 1985).

Two main models of receptor action have been developed for the analysis of the concentration-response data: (a) the null method and (b) the operational method. The null hypothesis is based on the idea that when two agonists produce the same response in the same tissue, a null equation can be used to predict the relation between the two drugs (Mackay, 1988). The operational model by Black and Leff (1983) uses a particular mathematical formula to form a relation between the response or effect (E) of an agonist to the concentration of agonist-receptor complexes (AR). This relation can be defined as:

$$\frac{E}{E_m} = \frac{[AR]}{K_E + [AR]} \qquad \text{(Black and Leff, 1983)} \qquad (4)$$

where K_E is the value of [AR] which produces half the maximal effect (E_m). The fraction of receptors occupied by the agonist is:

$$[AR] = \frac{[R_t]\,[A]}{K_A + [A]} \qquad \text{(Black and Leff, 1983)} \qquad (5)$$

where R_t is the total receptor concentration and K_A is the agonist-receptor dissociation constant. This equation can then be used to eliminate AR from equation (4) and leads to the predicted relation between E and [A] as:

$$\frac{E}{E_m} = \frac{\tau [A]}{(K_A + [A]) + (\tau[A])} \quad \text{(Black and Leff, 1983)} \quad (6)$$

where τ is the ratio R_t/K_E and is the measure of the efficiency of coupling of AR to E and is referred to as the operational efficacy. Although both the null and operational models estimate the affinity and efficacy of the receptor-agonist interaction, the advantage of one model over the other has been considered (Mackay, 1989, 1988; Leff, 1988).

Recently, a great deal of evidence has accumulated that many receptors are coupled to G-proteins. In these systems a ternary complex kind of mechanism has been postulated for agonist action (DeLean et al, 1978). The complex relationship can be described as the following:

$$H + R \overset{K_{1H}}{\rightleftharpoons} HR + T \overset{K_{2H}}{\rightleftharpoons} HRT \rightarrow \rightarrow \text{response} \quad \text{(Mackay, 1990)} \quad (7)$$

where H, R and T respresent the hormone, receptor and transducer molecules, respectively. K_{1H} is the apparent dissociation constant of the hormone-receptor complex and K_{2H} is the apparent dissociation constant of the HR complex for the effector molecule. The K_{2H} parameter can be predicted if the EC_{50} of an agonist is known and the dissociation constant (K_{1H}) and R_t are calculated from binding experiments since:

$$EC_{50} = \frac{K_{1H}}{1 + R_t / K_{2H}} \quad \text{(Black and Leff, 1983)} \quad (8)$$

According to this model, the magnitude of a physiological response by the hormone depends on the formation of the ternary HRT complex. The formation of the HRT is dependent on the concentration of H, R and T. Thus, the transducer molecule (eg. G-protein) is another site at which responses to an agonist are regulated.

2.2.3 CRITERIA FOR RECEPTOR VALIDATION

The radioligand binding assay has become a popular tool in endocrine research because it is simple to perform and useful information is provided. This assay permits the identification of binding site number, kinetic characteristics and pharmacological properties as well as relationships to other membrane components (Enna, 1984). However, it is imperative that certain requirements be met for the binding sites to be recognized as receptors (Laduron, 1984). These criteria include:

(a) The binding sites should be present in finite numbers (saturability). Saturability is only a minimal requirement for identification of a receptor since there are many examples of saturable binding to non-biological material (see Burt, 1985).

(b) Binding of the radioligand complex should be rapidly reversible following the addition of an excess amount of nonradioactive molecule of the same (or related) compound (reversibility). However, the action of certain ligands or drugs may be irreversible due to chemical modifications and thus, binding in these cases will not be reversible.

(c) Binding should be found only where receptors are expected and not

elsewhere (regional, subcellular and tissue distribution).

(d) Specificity of the binding site should be evident *i.e.* drugs belonging to similar chemical or pharmacological classes should compete for the binding site (competition) and drugs belonging to different classes should not.

(e) Pharmacology is a key criterion for receptor identification of binding. The rank order of potency of drugs or ligands in binding assays should correlate with the potencies in producing a biological response. However, this correlation will be true of antagonists but for agonists the potency may be affected by the parameter efficacy.

(f) Binding should be destroyed at extremes of temperature and pH and a linear correlation between tissue concentration and binding should be evident (Burt, 1985; Laduron, 1984).

2.2.4 TYPES OF BINDING ASSAYS

The fundamental characteristics of binding sites that are determined by ligand binding assays are apparent dissociation constant (K_d) and maximal binding capacity (B_{max}). Three basic types of experiments can be performed:

(i) *Saturation experiments* in which the total ligand (H_t) is increased and the amount specifically bound (HR) is determined at equilibrium (Bylund, 1980).

(ii) *Inhibition (competition) experiments* in which HR is measured as the concentration of unlabelled hormone is increased. H_t is held constant (Bylund, 1980).

(iii) *Kinetic experiments* in which HR is determined as a function of time with H_t held

constant. Association (on-rate) and dissociation (off-rate) experiments are included in this type of experiment (Bylund, 1980).

The following section will briefly review the analysis of these three types of experiments.

2.2.5 ANALYSIS OF BINDING DATA

2.2.5.1 *Saturation Experiments*

The major purpose of binding experiments is to provide information on the progressive saturation of binding sites by the ligand of interest (Klotz, 1983). The graphical representation of the binding isotherm plots the amount bound (B) against an increasing concentration of free radioligand (F). Various mathematical models have been used to linearize the data to make binding easier to interpret. However, the most popular approach for data analysis has been the Scatchard plot (Rosenthal, 1967; Scatchard, 1949) which plots the amount of ligand bound divided by the concentration of free ligand on the ordinate (B/F) versus the bound ligand concentration (B) on the abscissa (Munson, 1984; Klotz, 1982).

The basis of a Scatchard plot is as follows:

If the total concentration of receptors is B_{max}, then

$$[HR] + [R] = B_{max} \quad (9)$$

$$\text{or } [R] = B_{max} - [HR] \quad (10)$$

where [HR] represents the hormone bound to the receptor and [R] represents the

free concentration of the receptor at equilibrium.

The apparent dissociation constant for the hormone-receptor interaction can be described as:

$$K_d = [H][R]/[HR] \quad (11)$$

Since [HR] represents the amount of hormone bound to receptors at equilibrium (B) and [H] represents the concentration of free hormone (F), these variables can be substituted into equation (11):

$$K_d = [F][R]/[B] \quad (12)$$

Rearranging the equation:

$$[B]/[F] = [R]/K_d \quad (13)$$

Substituting equation (10) into equation (13):

$$[B]/[F] = \frac{B_{max} - [HR]}{K_d} \quad (14)$$

Rearranging equation (14):

$$B/F = -[B]/K_d + B_{max}/K_d \quad (15)$$

gives the equation of a straight line ($y = mx + b$). The receptor affinity expressed in terms of the K_d is the negative reciprocal of the slope of the line ($m = -1/K_d$; $K_d = -1/m$). At infinite concentrations of free hormone all receptors are occupied (B_{max}) so B/F approaches zero and the line cuts the abscissa ($B/F=0$; $y=0$). Thus, the total receptor concentration (B_{max}) is determined by the intercept of the line joining the points extrapolated to the x-axis (Hrdina, 1986; Bennett and Yamamura, 1985).

The graphical method for calculation of binding parameters introduced by Scatchard (1949) has become very popular. However, many assumptions must be made in the use of Scatchard analysis and a number of conditions satisfied for correct parameter estimates. These have been discussed in detail by Bolander (1989) and Bennett and Yamamura (1985) and include the assumptions that (a) the radiolabelled ligand behaves identically to the native hormone, (b) the radiolabelled ligand and receptor are both homogeneous, (c) data are collected at equilibrium, (d) no cooperativity is present (*i.e.* binding of the ligand to receptor does not affect subsequent binding), (e) non specific binding (NSB) is clearly defined and measured and (f) the receptor is unoccupied by endogenous hormone.

Once data have been collected for Scatchard analysis several problems with interpretation can occur. These include (a) subjectivity of drawing a straight line through a scatter of data points in the Scatchard plot, (b) application of an inappropriate or incorrect model to the data and (eg. fitting a curved Scatchard plot with a straight line),(c) ascribing too much significance to parameters like B_{max} that are estimated by extrapolation of the curve beyond the data points (Munson, 1984). Thus, questions have been raised by several researchers as to the reliability of binding parameters derived from Scatchard analysis (Bürgisser, 1984; Klotz, 1983, 1982).

In order to alleviate the problems with Scatchard analysis and the tediousness of analyzing binding data by hand, computerized programmes for complex binding

models based on the Law of Mass Action have been developed. A computerized, nonlinear, least squares regression analysis of untransformed data is an alternative approach to the Scatchard analysis (Bürgisser, 1984) and several convenient programmes are available (eg. BDATA, EMF software). The advantages of computer assisted data analysis over conventional methods have been well-documented (Bürgisser, 1984; Munson, 1984) and include:

(a) avoidance of the temptation to fit the data points to a straight line (Bürgisser, 1984).

(b) a number of binding models can be considered and fitted separately and the programme most consistent with the data can be selected (eg. 1 site versus 2 sites) (Munson, 1984).

(c) statistical tools are provided which are then used to make a statistical comparison between models for the best fit of the data (Munson, 1984).

2.2.5.2 *Competitive Inhibition Experiments*

Competitive inhibition binding studies involve incubation of a fixed concentration of radioligand with increasing concentrations of unlabelled ligand. The concentration of unlabelled ligand [L] that displaces 50 percent of specific binding (IC_{50}) can then be determined. The simplest method to determine the IC_{50} for a given ligand is the standard dose-response semi-log plot. The log of the concentration of the unlabelled ligand is plotted against the percent radioligand bound (Bylund,

1980).

The following equation can then be used to estimate an inhibition constant (K_i) of unlabelled ligand from the experimentally determined IC_{50}:

$$K_i = \frac{IC_{50}}{1 + [L]/K_d}$$ (Hrdina, 1986) (16)

2.2.5.3 *Kinetic Experiments*

The equilibrium constant can also be calculated from experiments in which binding is determined as a function of time. The rate constants of association (k_1) and dissociation (k_2) of ligand-receptor interactions are estimated and the K_d is equal to the ratio of k_2/k_1 since:

$$K_d = [L][R]/[LR] = k_2/k_1$$ (Bennett and Yamamura, 1985) (17)

The dissociation rate constant is estimated by incubating the tissue preparation with a fixed ligand concentration until equilibrium is reached, then adding an excess of nonradioactive analog or ligand. Specific binding is measured at various time intervals after the addition of displacer. The half life (t½) of loss of specific binding is estimated by a plot of log [B] against time. The dissociation rate constant is calculated as .693/t½. Alternatively, the slope of the line generated by log[B] against time is multiplied by -2.303 to estimate k_2 (Hrdina, 1986; Bennett and Yamamura, 1985).

The association rate constant can be estimated by measuring the amount of

specifically bound ligand at various time intervals (B_t) until equilibrium is reached (B_{Eq}). A plot of $\ln[B_{Eq}/(B_{Eq}-B_t)]$ versus time will have a slope of k_{obs}. The association rate constant can then be calculated from the equation:

$$k_1 = (k_{obs} - k_2)[L]^{-1} \qquad \text{(Hrdina, 1986)(18)}$$

This method is used to estimate k_1 when the amount of free ligand bound is less than 10 percent of the total radioactive ligand added. Within experimental error, the K_d derived from kinetic experiments (k_2/k_1) should be similar to that obtained from saturation analysis (Hrdina, 1986; Bennett and Yamamura, 1985).

2.3 THE GnRH RECEPTOR

2.3.1 GnRH ANALOGS

Early studies of GnRH receptors attempted to characterize binding using tritiated [^{3}H] or radio-iodinated [^{125}I] GnRH as radioligands. However, the specific activity of ^{3}H-GnRH was low (Clayton and Catt, 1981a) and ^{125}I-GnRH was susceptible to significant degradation, even at 4°C (Clayton et al, 1979b). The synthesis of analogs of natural sequence GnRH which are resistant to enzymatic degradation overcame these earlier problems (Karten and Rivier, 1986). The presence of a D-amino acid in place of the sixth residue yields metabolically stable analogs resistant to enzymatic degradation (Zohar et al, 1990; Ccy et al, 1976). Combining this modification with an ethylamide substitution of glycine in position 10 enhances the affinity of the analog for the GnRH binding site (Fujino et al, 1974).

Examples of these types of analogs include DSer^{6}EA, DLeu^{6}EA and DAla^{6}EA with potencies 10 to 100 times greater than native GnRH (Clayton and Catt, 1980; Perrin et al, 1980). These analogs interact with selectivity for specific pituitary GnRH binding sites (see review by Clayton and Catt, 1981a) and are largely responsible for the present understanding of the mechanism of GnRH at a molecular level.

2.3.2 LOCALIZATION AND IDENTIFICATION

GnRH binding sites detected by radioligand binding assays are located on the plasma membrane of pituitary gonadotropes (Marian and Conn, 1983; Clayton et al, 1978). Some reports have indicated intracellular GnRH binding in lysosomal, secretory granules and nuclear fragments but these appear to be contamination by plasma membrane (Marian and Conn, 1983). A GnRH binding component with a molecular weight of 60,000 has been demonstrated in the rat pituitary by electrophoresis using a radiolabelled photoaffinity ligand to label GnRH (Hazum, 1983; Hazum and Keinan, 1982). This molecular weight was confirmed by Eidne et al (1985) using a ligand-immunoblotting technique. However, using target size analysis Conn and Venter (1985) estimated a molecular weight for the GnRH binding site of approximately 136,000. It has been postulated that the 60,000 Dalton band is a subunit of the GnRH receptor (Conn et al, 1987; Conn and Ventor, 1985) and that one component is the binding protein and the other a regulatory protein (Hazum and Conn, 1988).

The pituitary has been identified as the main target organ for GnRH. However, extrapituitary GnRH binding has been identified in rat gonadal tissue (reviewed by Hsueh and Jones, 1983) and rat hippocampus (Badr et al, 1988; Reubi et al, 1987). Specific GnRH binding sites have been reported in human corpus luteum (Bramley and Menzies, 1986), human granulosa cells (Latouche et al, 1989) and placenta (Belisle et al, 1984). The role of GnRH binding sites in these tissues remains to be elucidated. A GnRH-like peptide produced by the ovary has been purified and the authors have hypothesized that this substance (gonadocrinin) may be involved in the hypothalamic-pituitary feedback system (Ying et al, 1981).

2.3.3 BIOCHEMISTRY OF THE GnRH RECEPTOR

Biochemical characteristics of the binding properties of the GnRH receptor have recently been investigated. GnRH binding sites are glycoproteins containing a sugar moeity (probably sialic acid) that participates in the binding of GnRH (Keinan and Hazum, 1985). Phospholipids are apparently necessary for the structural integrity of the GnRH receptor since binding requires the presence of the exterior hydrophilic head groups and the fatty acid linked to the carbon of the phospholipid (Hazum et al, 1982a). Furthermore, two amino acid residues (tyrosine and tryptophan) and two carboxylic groups of the receptor are probably essential for formation of the hormone-receptor complex (Keinan and Hazum, 1985). The spatial conformation of the amino acid arginine in position 8 (positively charged) and the carboxyl groups on

the binding site (negatively charged) is believed to be the driving force for the formation of the hormone-receptor complex (Hazum, 1987; Keinan and Hazum, 1985).

2.3.4 INTRACELLULAR PATHWAY OF THE GnRH RECEPTOR

2.3.4.1 *Receptor Mediated Endocytosis of GnRH*

Pituitary GnRH binding sites located on the plasma membranes have been shown to be initially evenly distributed on the cell surface, then formed into clusters or aggregates (Hopkins and Gregory, 1977) which then become internalized (Schvartz and Hazum, 1987). In vivo uptake studies using a radiolobelled GnRH agonist revealed significant detection of the label in lysosomal and Golgi apparatus (Duello et al, 1983; Jennes et al, 1983). Similarly, incubation of dispersed rat pituitary cells with a photoaffinity GnRH-label resulted in half the label being associated with the plasma membrane while the rest was present in lysosome-like structures (20%), secretory granules (21%) and the Golgi complex (9%)(Hazum et al, 1985; Hazum et al, 1982b). These results suggest that GnRH agonists, following binding to specific binding sites, may be taken up by receptor mediated endocytosis and transported to Golgi and lysosomal structures.

2.3.4.2 *Degradation and Recycling of GnRH Receptors*

The GnRH hormone receptor complex appears to be degraded following

endocytosis and the nature of the site of degradation is postulated to be lysosomal (Schvartz and Hazum, 1987). In addition, a proportion of GnRH binding sites may be recycled back to the cell surface (Schvartz and Hazum, 1987). These intracellular pathways have been identified by the use of photoaffinity labelling of GnRH receptor complexes after internalization. However, the photoaffinity technique results in a covalent attachment of GnRH to the binding site which cannot dissociate (Hazum and Conn, 1988). Hence, it is unclear whether or not these covalently bonded receptors are metabolized in the same way (Hazum and Conn, 1988).

2.3.4.3 *LH Release and GnRH Intracellular Interactions*

The available evidence indicates that GnRH receptor internalization is not required for GnRH-stimulated LH release (Gorospe and Conn, 1987; Conn and Hazum, 1981; Conn et al, 1981b). In studies in which receptor internalization was prevented, gonadotropin secretion was not affected (Conn et al, 1981b; Conn and Hazum, 1981). Removing GnRH from the extracellular medium of dispersed pituitary cells after internalization resulted in the return of LH to basal levels (Conn et al, 1986). Thus, a role for internalization in gonadotropin release has been excluded since internalization was not necessary for LH response nor was it sufficient in the absence of exogenous GnRH. The physiological significance of the internalization of GnRH and its binding site, if any, is not clear.

2.3.5 BINDING PROPERTIES

Table 1 provides a summary of the available literature on binding properties of GnRH to the pituitary. In general, GnRH appears to bind to a single class of high affinity binding sites on pituitary membranes. A good correlation between apparent dissociation constants and biological activity for a number of agonists has been reported, suggesting the physiological relevance of the binding sites (Habibi et al, 1989; Loumaye et al, 1982).

2.4 THE EFFECTOR MECHANISM OF GnRH

2.4.1 OVERVIEW

The signalling system utilizing cAMP (adenosine 3',5'-cyclic monophosphate) as a second messenger is perhaps the best known, yet another pathway employing inositol lipids as part of the transduction mechanism has recently been identified. A number of agonists stimulate phosphoinositol metabolism and a second messenger function for two of the inositol lipid breakdown products, inositol trisphosphate and diacylglycerol, has been proposed (Berridge and Irvine, 1984).

Figure 1 provides a schematic view of the major pathways for phosphoinositide degradation, biosynthesis and the formation of phosphoinositide-derived second messengers. Briefly, phosphatidylinositol (PI) is phosphorylated at the plasma membrane to phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-

TABLE 1: Binding properties of GnRH analogs to pituitary membranes.

Species	Sex	K_d (nM)	B_{max} (fmol/mg)	Reference
rat	F	NR	27	Abbot et al (1986)
	F	NR	175 (M); 187 (E) 361 (D,P)	Rosen et al (1989)
	F	0.1-0.2	282-438	Marian et al (1981)
	F	0.7-0.12	177	Attardi and Happe (1986)
	F	0.2	102 (E); 270 (P) 120 (D)	Marchetti and Cioni (1988)
	F	0.2	105 (E); 200 (D)	Clayton et al (1980a)
	M	0.2	112	Clayton et al (1980a)
	M	NR	545	Rosen et al (1988)
	M	0.16	150-180	Clayton and Catt (1981b)
	M	0.3	NR	Perrin et al (1983)
	M	0.21	235	Clayton a. 1 Catt (1980)
ovine	F	0.13	$2.9/10^{-7}$ cells	Laws et al (1990)
rabbit	M	0.36	110	Limonta et al (1986)
	F	0.14	188	Thorson et al (1985)

P = proestrus; D = diestrus; E = estrus; M = metestrus; NR = not reported

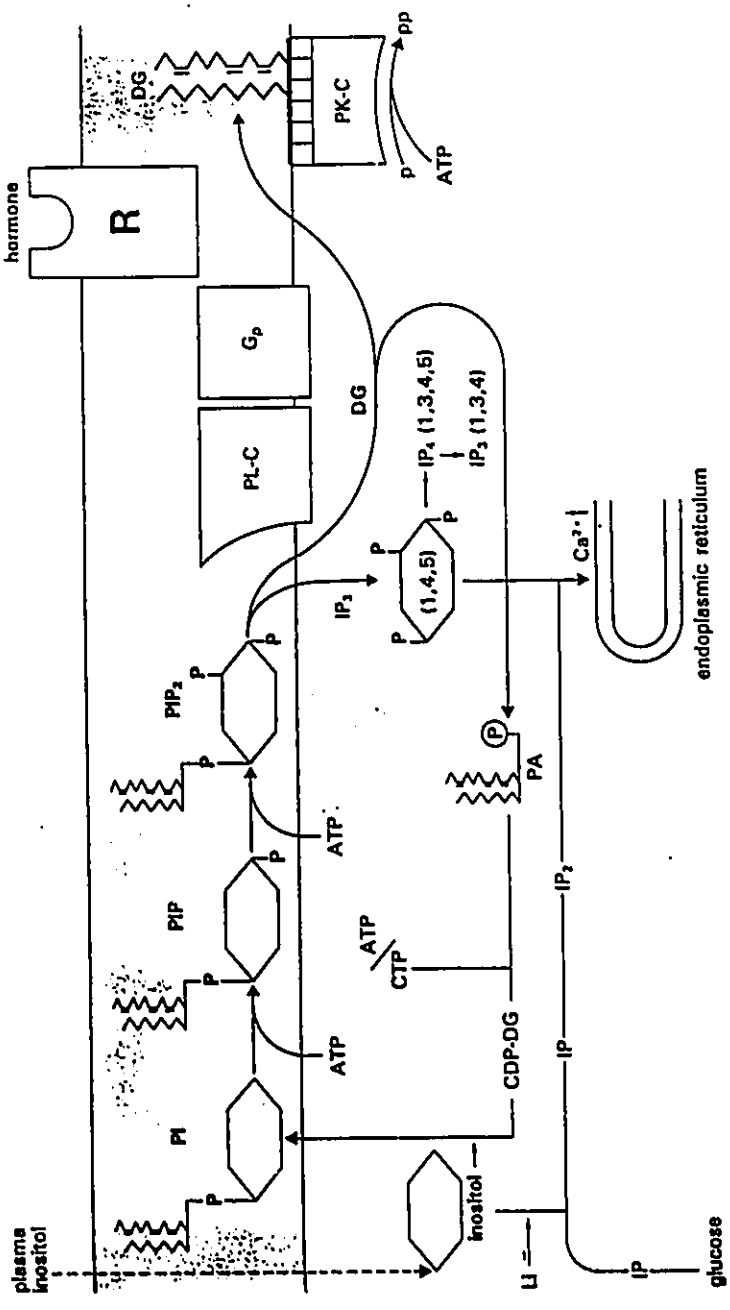


FIGURE 1: Generation of phosphoinositide-derived messenger molecules (from Braulieu and Kelly, 1990)

bisphosphate (PIP_2) in the presence of specific kinases. PIP and PIP_2 are interchangable in the plasma membrane through the action of the two kinases and two phosphatases. The hydrolysis of PIP_2 to 1,2-diacylglycerol (DG) and inositol trisphosphate (IP_3) occurs via an agonist-stimulated phosphoinositide-specific phospholipase C (PLC). These two products (DG, IP_3) are capable of functioning as second messengers. IP_3 appears to act by mobilizing intracellular calcium whereas DG activates protein kinase C. DG is metabolized by lipases to release monoacylglycerol (MG) and arachidonic acid (AA) for prostaglandin (PG) synthesis or is phosphorylated via diacylglycerol kinase to regenerate phosphatidic acid (PA). IP_3 is rapidly degraded via inositol phosphatases into free myo-inositol and inorganic phosphate. Myo-inositol and DG are then utilized for resynthesis of phosphatidylinositol (Majerus et al, 1988; Berridge, 1987; Abdel-Latif, 1986; Berridge, 1984).

2.4.2 CALCIUM AND GnRH ACTION

Early studies attempted to associate adenylate cyclase with the action of GnRH but the changes in cAMP were found to be uncoupled from LH release (Conn et al, 1979). More and more evidence has linked calcium (Ca^{2+}) to GnRH stimulated LH release (see reviews by Naor, 1990a; Huckle and Conn, 1988). Three general lines of investigation demonstrate the importance of calcium in signal transduction of GnRH and identify calcium as the second messenger in GnRH

action.

(a) Extracellular Ca^{2+} is required for GnRH-stimulated LH release. GnRH-stimulated LH release was abolished when pituitary cells were placed into Ca^{2+}-free medium (Izumi et al, 1989) or medium containing extracellular Ca^{2+} chelators EDTA (Izumi et al, 1989; Jamaluddin et al, 1989) or EGTA (Hansen et al, 1987). The addition of Ca^{2+} channel blockers such as verapamil (Jamaluddin et al, 1989), methoxyverapamil (Hansen et al, 1987; Bates and Conn, 1984) or nifedipine (Tasaka et al, 1988) produced similar effects demonstrating that extracellular Ca^{2+} is essential for GnRH-stimulated LH release.

(b) Elevation of intracellular Ca^{2+} produces LH release. The addition of agents that increase intracellular Ca^{2+} (e.g. Ca^{2+} ionophores) cause LH release with efficacies similar to that of GnRH (Beggs and Miller, 1989; McArdle et al, 1987).

(c) An increase in intracellular Ca^{2+} occurs in response to GnRH. A rapid (8-10 seconds) increase in cytosolic pituitary Ca^{2+} following GnRH treatment has been demonstrated in studies using Ca^{2+}-sensitive fluorescent probes (Chang et al, 1986; Clapper and Conn, 1985).

2.4.3 ROLE OF CALMODULIN

Calmodulin (CaM) is an intracellular Ca^{2+} receptor that alters the activity of many Ca^{2+}-dependent enzymes. The administration of GnRH has been shown to increase plasma membrane CaM levels (as measured by RIA) and decrease cytosolic

CaM in a dose and time-dependent manner (Conn et al, 1981a). These results are consistent with the translocation of CaM from the cytoplasm to the plasma membrane. CaM antagonists inhibit GnRH-stimulated LH release from pituitary cultures (Harris et al, 1985). However, it is not yet known which proteins or enzymes CaM regulates. Recently, several possible CaM-binding component subunits have been identified with molecular weights of 205, 200, 135, 60 and 52 (Wooge and Conn, 1988). Thus, it seems possible that GnRH provokes an increase in intracellular Ca^{2+} which binds to CaM to produce a CaM-calcium complex which then regulates a number of intracellular enzymes (Conn et al, 1987).

2.4.4 GnRH AND ALTERED PHOSPHOLIPID METABOLISM

Interest has recently focused on the potential physiological role of phospholipid (PL) metabolism in the stimulus-response coupling of GnRH. [^{32}P]orthophosphate ([^{32}P]P_i) incorporation into PA, PI, PIP and PIP_2 occurred rapidly (1-2 minutes) following exposure to GnRH (Morgan et al, 1987; Andrews and Conn, 1986). The labelling of other membrane PLs such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylserine (PS) were not significantly affected by GnRH (Andrews and Conn, 1986). The authors inferred that the specificity and rapidity of stimulation of inositol PL metabolism suggests an involvement in the initial event of GnRH action. Thus, following GnRH binding to its receptor, PL hydrolysis results in substrates that would be available for DG and

IP_3 formation.

2.4.5 GnRH AND THE FORMATION OF PHOSPHOINOSITIDE METABOLITES

The inositol phospholipid hydrolysis products include DG, inositol 1-phosphate (IP), inositol 1,4-bisphosphate (IP_2) and inositol 1,4,5-trisphosphate (IP_3). Stimulation of phospholipase C by GnRH would be expected to increase the production of phosphoinositide metabolites. Total [^{3}H]-inositol phosphates accumulate in response to GnRH (Watanabe et al, 1990). However, these measurements reflect a sum of inositol tris-, bis-, and mono-phosphates. Information concerning the relative proportion of each of the PL metabolites is not provided and thus the results are limiting as to identification of a second messenger.

Stimulated IP metabolism has also been investigated by measuring the production of individual PI metabolites. The inositol phosphates (IPs) IP, IP_2 and IP_3 rapidly increase in response to GnRH in the pituitary (Naor, 1990; Morgan et al, 1987; Kiesel et al, 1986; Schrey, 1985). Time periods of IP accumulations have been similar amongst studies, with rapid increases in IP_3 within 10 seconds (Naor et al, 1986), 30 seconds (Kiesel et al, 1986) and 2-5 minutes (Schrey, 1985). The initial rate of formation of IP_3 was greater than the rate of formation of IP_2 or IP suggesting that an early effect of GnRH is the hydrolysis of PIP_2 (Kiesel et al, 1986).

IP_3 has been proposed to be the second messenger involved in calcium mobilization from intracellular stores (presumably the endoplasmic reticulum)(Putney, 1987; Berridge, 1984). Thus, GnRH-induced increases in IP_3 would be expected to precede or accompany increases in Ca^{2+}. GnRH has repeatedly been shown to increase cytosolic free Ca^{2+} (Chang et al, 1986; Leung et al, 1986; Clapper and Conn, 1985). The early component of GnRH-stimulated LH release has been unaffected by changes in, or depletion of, extracellular Ca^{2+} (Hansen et al, 1987). Similar dose and temporal relationships between IP_3 formation and Ca^{2+} mobilization in response to GnRH provides support for the role of IP_3 as a second messenger (Davis et al, 1986). However, there is a need to conduct experiments which introduce IP_3 into specific cells to investigate the correlation between the effects of GnRH and IP_3 on Ca^{2+} release.

2.4.6 PROTEIN KINASE C

As mentioned previously, the two products of PL breakdown (IP_3, DG) function as second messengers. IP_3 mobilizes intracellular Ca^{2+} while DG activates protein kinase C (PKC). Several lines of investigation have demonstrated that the action of GnRH involves PKC activation (see reviews by Naor, 1990a; Huckle and Conn, 1988).

The first line of evidence suggesting a role for PKC in GnRH action came from studies in which PKC appeared to mimic the effects of GnRH. Phorbol esters,

tumor promotors that activate PKC, have repeatedly been shown to stimulate LH release (McArdle et al, 1988; Harris et al, 1985). A PKC inhibitor, staurosporine, inhibited both GnRH and phorbol ester-stimulated LH release (Dan-Cohen and Naor, 1990). Similar stimulatory effects on LH release resulted from the addition of synthetic DGs (Harris et al, 1985). Thus, phorbol esters or synthetic DGs stimulate LH release by mimicking the effects of endogenous DG in activating PKC.

A second approach in identification of the role of PKC in GnRH action has been the measurement of the endogenous production of PKC. PKC levels in rat pituitary cultures were elevated following GnRH administration (Andrews et al, 1988). A GnRH-induced redistribution of PKC from the cytosol to PL DG-rich tissue fragments has been reported (McArdle and Conn, 1986). Since the mechanism of PKC involves redistribution of the enzyme, a role for PKC in GnRH action has been postulated.

Nevertheless, the possible role of PKC in LH release remains controversial. Phorbol esters stimulate (Harris et al, 1985) and phorbol ester inhibitors block (Dan-Cohen and Naor, 1990) LH release with less efficacy than GnRH itself suggesting a dissociation between GnRH and PKC. In PKC-depleted cells, LH release in response to GnRH was not inhibited (McArdle et al, 1987a). It has been suggested that GnRH action may involve a bifurcating messenger system consisting of (i) elevation of Ca^{2+} by IP_3 and (ii) activation of PKC (Naor and Eli, 1985). These second messengers would produce a synergistic effect necessary for eliciting the GnRH response (Naor,

1990a; Harris et al, 1985).

2.5 REGULATION OF THE GnRH RECEPTOR

2.5.1 ENDOCRINE STATUS

Various endocrine states alter pituitary GnRH binding site number while binding affinity remains constant (Clayton and Catt, 1981). Both testosterone (Giguere et al, 1981) and estradiol (E_2)(see Clayton et al, 1985) administration reduce binding site number. Conditions such as lactation (Lee et al, 1989) and aging (Marchetti and Cioni, 1988; Marian et al, 1981) also result in a decline in GnRH binding site number. Since these conditions reflect changing LH levels, it is generally accepted that GnRH binding site numbers are predictive of serum gonadotropin levels. However, several reports have demonstrated a dissociation between GnRH binding sites and serum LH (Naik et al, 1985; Leung et al, 1984; Adams and Spies, 1981) suggesting that factors other than binding site number are important in the release of gonadotropins.

Changes in GnRH binding site number during the estrous cycle of the rat have been investigated in an attempt to explain the mechanism of the LH surge. GnRH binding site levels are low during estrus and metestrus (Savoy-Moore et al, 1981) but gradually increase in early (see Lloyd and Childs, 1988) or late (Marian et al, 1981) diestrus. A close temporal relationship exists between the increases in E_2 and GnRH binding site number, suggesting a steroid induction of GnRH receptors

prior to the LH surge. It is possible that the increase in GnRH receptor number enables the pituitary to respond to low concentrations of GnRH with a continuous increase in LH secretion. Thus, the E_2-induced change in GnRH binding site number may be involved in the mechanism of the LH surge.

2.5.2 AUTOREGULATION

GnRH is one of the few hormones that regulates its own receptor. Intermittent administration of GnRH to intact rat pituitaries (Young et al, 1985) or GnRH-deficient mice (Detta et al, 1984) results in an increase in GnRH binding site number. A post-gonadectomy rise in GnRH binding site number occurs and this can be prevented by the adminstration of a GnRH antagonist (Clayton et al, 1985) or anti-GnRH serum (Frager et al, 1981). Thus, the correlation between GnRH and GnRH binding site levels suggests that GnRH regulation of its own receptor may be one way in which the activity of the gonadotrope is determined (Clayton, 1989).

2.5.3 GONADOTROPE DESENSITIZATION AND GnRH RECEPTOR DOWN REGULATION

The binding of GnRH and its analogs can produce homologous desensitization in the pituitary gonadotrope (Conn et al, 1984; Badger et al, 1983). This phenomenon has been observed both in vivo and in vitro following long term (1-12 hours) exposure to GnRH or its analogs (Smith and Conn, 1981; Smith and Vale, 1981). In

contrast, pulsatile administration (one pulse of a few minutes per hour) prevents or delays the occurrence of desensitization (Badger et al, 1983).

Down regulation of receptors is believed to result from physical internalization of the agonist-occupied receptor (Hazum and Conn, 1988; Hazum et al, 1980). Hence, the resultant reduction in the ability of GnRH to provoke LH release is due in part to the loss of cell surface receptors (Huckle and Conn, 1988). Neither receptor down regulation nor desensitization requires extracellular Ca^{2+} (Smith and Conn, 1983) or PKC (McArdle et al, 1987a).

2.5.4 DEVELOPMENTAL STAGE

Pituitary GnRH binding site numbers vary during sexual maturation while binding affinity remains constant (Chan et al, 1981; Dalkin et al, 1981). In male rats, pituitary GnRH binding site number increased from 5 days of age (424 ± 64 fmol/mg protein) to a peak at 30 days (594 ± 54 fmol/mg protein)(Dalkin et al, 1981). In female rats, a similar profile existed but peak values of GnRH binding site numbers (720 ± 52 fmol/mg protein) were evident by 20 days of age (Dalkin et al, 1981). A secondary increase in GnRH binding site number in the female pituitary was observed at 50 days (Duncan et al, 1983) or 70 days (Chan et al, 1981). Similar results for both male and female rats were reported by Chan et al (1981). Following the observed peak, GnRH binding sites declined gradually to a plateau level by 60 days and 50 days of age for male and female rats, respectively (Chan et al, 1981). In

contrast, Duncan et al (1983), expressing the measurement of GnRH receptors as fmol/pituitary gland, reported that the number of pituitary GnRH binding sites in the male rat remained stable following the rise at 35 days.

The role of the pituitary GnRH receptor during sexual maturation has been investigated by determining the relationship between GnRH binding site and serum gonadotropins and/or response to GnRH. The concentration of pituitary GnRH binding sites in the male and female rat were maximal in the prepubertal period when serum gonadotropins LH and FSH were elevated (Chan et al, 1981). The change in pituitary GnRH binding sites in the female (Dalkin et al, 1981) and male (Chan et al, 1981; Dalkin et al, 1981) followed a pattern similar to that of FSH. Since the initial response to GnRH in the rat was an increase in FSH secretion (Aubert et al, 1979), the authors concluded that the high concentration of FSH and GnRH binding sites prior to puberty probably results from an increase in GnRH secretion from the hypothalamus (Dalkin et al, 1981). Furthermore, the highest concentration of GnRH binding sites in the pituitary of male and female rats correlated with maximal responsiveness to GnRH (Dalkin et al, 1981). This temporal relationship between GnRH binding and responsiveness to GnRH led to the suggestion that GnRH plays a role in the mechanism of pituitary responsiveness during sexual development (Dalkin et al, 1981). The high prepubertal concentration of pituitary GnRH binding sites would result in increased gonadotropin release (Chan et al, 1981) leading to sexual maturation.

A role for steroids in the decline of pituitary GnRH receptors numbers following sexual maturation has been hypothesized (Duncan et al, 1983). The rise in gonadal steroids during maturation may exert a negative effect on GnRH secretion from the hypothalamus which in turn would decrease GnRH binding site number (Dalkin et al, 1981). Castration induces an increase in pituitary GnRH binding sites in the rat (Clayton and Catt, 1981a; Frager et al, 1981) demonstrating the negative effect of steroid hormones on GnRH binding sites. Female rats of all ages (5-60 days) showed a two-fold increase in GnRH binding sites five days post-castration (Duncan et al, 1983). Male rats, however, had a two-fold increase in GnRH binding site numbers following castration from ages 5-25 days and 50-60 days but no change occurred at ages 30-45 days (Duncan et al, 1983). Nevertheless, the experiment demonstrated that gonadal steroids have an effect on pituitary GnRH binding sites and this action may be mediated through a decline in hypothalamic GnRH secretion (Duncan et al, 1983). Thus, the changes in pituitary GnRH binding, serum gonadotropins and sex steroids suggest a functional role for the GnRH receptor and its regulation during sexual maturation (Clayton and Catt, 1981a).

2.6 OBJECTIVES OF THE PRESENT STUDY

The purpose of the present study was to investigate the role of the pituitary GnRH binding site in the process of sexual maturation of the female rabbit. A radioligand binding assay was established using the GnRH analog DAla^{6}EA to

characterize the pituitary GnRH binding site and measure the concentration of ^{125}I-DAla^{6}EA binding sites at various ages (25-120 days) of the female rabbit. A correlation between the previously reported increase in circulating LH at 30-50 days of age (YoungLai, 1986) and ^{125}I-DAla^{6}EA binding site number may indicate a functional role for GnRH binding sites in the female rabbit. Finally, whether or not the responsiveness of the pituitary to GnRH changes with respect to binding site number was investigated by determining the relationship between LH response to DAla^{6}EA and ^{125}I-DAla^{6}EA binding site number. The results of this study may provide insight into the mechanism by which the female rabbit becomes sexually mature.

CHAPTER THREE

MATERIALS AND METHODS

3.1 ANIMALS

New Zealand White (NZW) rabbits (2-2.5 kg) were obtained from local breeders (Maple Lane Farms, Clifford, Ontario) and maintained under constant temperature (21°C) and lighting conditions (12 hr light: 12 hr dark). Food and water were available *ad libitum*. For experiments requiring animals of defined ages, female New Zealand White rabbits were purchased at specified days after birth. Animal tissues for use in the initial characterization experiments were provided by Sargent Farms Ltd., Milton, Ontario.

3.2 PITUITARY MEMBRANE PREPARATION

Following euthanasia (65 mg sodium pentobarbital per ml; 1 ml per kg body weight injected into superficial ear vein), rabbit pituitaries were rapidly removed and the posterior pituitaries discarded. Anterior pituitaries were quickly frozen in liquid nitrogen and stored individually at -70°C until assayed. At this time pituitaries were thawed on ice and individually homogenized in 1 ml 0.32 M sucrose (10-15 strokes with a glass-glass homogenizer). The homogenate was centrifuged at 600 x g for 5 minutes and the resulting supernatant centrifuged at 14,000 x g for 15 minutes. The second pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) to a protein concentration of approximately 80-120 μg protein/100 μl. The membrane preparation was conducted at 4°C and used immediately in the radioligand binding assay. Protein content of pituitaries was determined by the method of Lowry et al (1951) (see

Appendix A).

3.3 MATERIALS

3.3.1 GnRH AND GnRH-PEPTIDES

The GnRH analog DAla^6-des-Gly^{10}-GnRH ethylamide (DAla^{6}EA) was purchased from Sigma Chemical Company (St. Louis, Mo., USA). The GnRH antagonist DpGlu^1 and GnRH agonist DLeu^{6}EA were gifts from Dr. J. Jarrell (University of Calgary). Gonadorelin Hydrochloride (Factrel) was purchased from Ayerst Laboratories (Montreal, Canada). Native GnRH was kindly donated by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (NIADDK)(NIH, Bethesda, Maryland).

3.3.2 MISCELLANEOUS MATERIALS

PGE_2, $PGF_{2\alpha}$, oxytocin and arginine vasopressin were gifts from Dr. D. Crankshaw (McMaster University). Polyethylenimine (PEI) was provided by Dr. L. Niles (McMaster University). Bacitracin, bovine serum albumin (BSA) and Dowex (1x8-400 chloride form) were purchased from Sigma Chemical Company (St. Louis, Mo., USA). Dithiothreitol and soybean trypsin inhibitor were supplied by M. Morency (McMaster University). Sodium pentobarbital (somnotol) was from MTC Pharmaceuticals (Cambridge, Ontario). Heparin was purchased from Leo Laboratories (Pickering, Ontario). Reagents for LH radioimmunoassay (RIA) were

provided by Dr. A.F. Parlow (LH standard WP360A), Dr. L.E. Reichert, Jr. (LH antigen LER-1056-C2) and Dr. Scaramuzzi (guinea-pig anti-rabbit LH 6F GPāLH). Tris (hydroxymethyl) aminomethane was purchased from Biorad (Richmond, California, USA). ^{125}I-Na was purchased from Radiopharmacy, McMaster University. Microcentrifuge polypropylene tubes (1.5 ml) were obtained from Quality Scientific Plastics, USA.

3.4 RADIOIODINATION TECHNIQUE

Radioiodination of the GnRH analog DAla^{6}EA for use in the radioligand binding assay was carried out according to the chloramine-T (N-monochloro-p-toluenesulfonamide) method previously described by Clayton (1983). Briefly, the GnRH analog (5μg/10μl 0.1 M phosphate buffer) was labelled with ^{125}I (1.5 mCi) using the oxidizing agent chloramine-T (5mg/10ml 0.1 M phosphate buffer) and the reaction allowed to continue for 5 minutes at room temperature. The reaction was terminated by the addition of 400 μl 0.1 M phosphate buffer (pH 7.5). Unreacted ^{125}I was removed by the addition of 200 μg Dowex (1x8-400 mesh) followed by centrifugation (5 minutes at 600 x g). The resulting supernatant was applied to a Sephadex G-25 column (1 x 55 cm) and the analog eluted with 0.1 N acetic acid containing 0.25% BSA. Collection of 1.5 ml fractions resulted in peak labelled analog at approximately tubes 18-26. For further details see Appendix B.

3.5 HISTOLOGY OF RABBIT PITUITARIES

Hematoxylin and eosin staining was carried out on pituitaries of adult NZW rabbits. Following euthanasia (65 mg sodium pentobarbital per ml; 1 ml per kg body weight injected into superficial ear vein), pituitaries were quickly removed and placed in Davidson's Fixative overnight (24-36 hours). A series of dehydration steps (70% alcohol - 95% alcohol) was followed by infiltration with α-terpineol. The following day tissues were embedded in paraffin wax. Paraffin blocks were sectioned (10 μm) on a Spencer "820" rotary microtome (American Optical Co.) and slides were dried overnight on a warming plate. Slides were stained with hematoxylin and eosin using a standard histological procedure (see Appendix C).

3.6 GnRH BINDING ASSAY PROCEDURES AND EXPERIMENTS

3.6.1 STANDARD GnRH BINDING ASSAY CONDITIONS

The GnRH analog DAla^{6}FA was used as both tracer and unlabelled hormone. Approximately 80-120 μg protein of membrane preparation (14,000 x g pellet; 100 μl) was incubated in 1.5 ml polypropylene microcentrifuge tubes precoated overnight with 2% BSA/Tris-HCl. For experiments using a fixed labelled ligand concentration, approximately 0.4 nM ^{125}I-DAla^{6}EA in 100 μl buffer (Tris-HCl 0.1% BSA, pH 7.4) was added. For experiments to determine apparent dissociation constant (K_d) and maximal binding capacity (B_{max}), 100 μl aliquots of increasing concentrations of labelled ligand (0.1-10 nM) were used. Non-specific binding (NSB) was assessed in

tubes containing 10^{-6}M unlabelled DAla^{6}EA (100 μl). The incubation volume was made up to 400 μl with assay buffer (50 mM Tris-HCl, pH 7.4). All samples were assayed in duplicate or triplicate. Incubation occurred at 4°C for 60 minutes and was terminated by centrifugation (15 min at 11,000 x g). The supernatants were aspirated, pellets washed three times with 400 μl assay buffer and the radioactivity of the pellets counted in a γ-spectrometer (L-1261 MultiGamma Counter). NSB was subtracted from total binding to obtain specific binding.

3.6.2 CHARACTERIZATION OF ^{125}I-DALA^{6}EA BINDING

Standard assay conditions using a fixed labelled DAla^{6}EA concentration (approximately 0.4 nM; approximate K_d) with or without 10^{-6}M unlabelled DAla^{6}EA were modified as follows:

3.6.2.1 *Tissue Linearity*: To determine the correlation between tissue concentration and ^{125}I-DAla^{6}EA binding, increasing concentrations (20-240 μg protein) of rabbit pituitary membrane homogenate (14,000 x g pellet) were used.

3.6.2.2 *Temperature Dependence*: ^{125}I-DAla^{6}EA binding to rabbit pituitary membrane preparations (14,000 x g pellet) was conducted at 4°C, 21°C, 30°C and 37°C.

3.6.2.3 *Time Dependence*: Time-dependence of ^{125}I-DAla^{6}EA binding was studied

during incubation of pituitary membrane preparations at 4°C and 30°C. ^{125}I-DAla^{6}EA was incubated with pituitary membrane preparation for a maximum period of 120 minutes. At time periods 15, 30, 45, 60, 90 and 120 (4°C only) minutes the incubations were terminated.

3.6.2.4 *Effect of Bacitracin, Dithiothreitol (DTT) and Soybean Trypsin Inhibitor on ^{125}I-DAla^{6}EA Binding:* Rabbit pituitary membrane preparations were incubated with labelled DAla^{6}EA with parallel incubations containing final concentrations of bacitracin (100 μg/ml), DTT (0.1 M) or soybean trypsin inhibitor (1 mg/ml).

3.6.2.5 *Maximal Bindability*: To determine the maximal bindable fraction of ^{125}I-DAla^{6}EA, a low concentration (10^{-11}M) of radiolabelled DAla^{6}EA was incubated with increasing amounts of rabbit pituitary membrane preparation (5-310 μg protein). The percentage of ^{125}I-DAla^{6}EA added that was specifically bound was determined following correction for NSB.

3.6.2.6 *Competition Curves*: Pituitary membrane preparations were incubated with a constant amount of ^{125}I-DAla^{6}EA (approximately 0.4 nM) in the presence of increasing concentrations of cold DAla^{6}EA, Factrel, DLeu^{6}EA, native GnRH or DpGlu^1 (10^{-11}M to 10^{-6}M). Total tubes containing labelled DAla^{6}EA and membrane only served as a control. Triplicate determinations were made at each concentration.

A non-linear curve-fitting computer programme (CDATA) was used to estimate the IC_{50}s fitted to a one or two binding site model. For agents unrelated to GnRH, a single point containing a large amount (10^{-6}M) of arginine vasopressin, PGE_2, $PGF_{2\alpha}$, LH, FSH, TSH and oxytocin was used.

3.6.2.7 *Effect of pH*: The effect of pH on ^{125}I-DAla^{6}EA binding to rabbit pituitary membrane preparations was investigated over a range of 4 to 10. pH was adjusted by the addition of 0.1 N HCl or Tris Base to the assay buffer.

3.6.2.8 *Tissue Specificity*: ^{125}I-DAla^{6}EA binding to adult rabbit membrane preparations (14,000 x g pellet) of hypothalamus, ovary, kidney, spleen, skeletal muscle and liver were measured at a fixed ligand concentration with and without 10^{-6}M unlabelled DAla^{6}EA.

3.6.2.9 *Saturation*: Pituitary membrane preparations were incubated with increasing concentrations of labelled DAla^{6}EA (0.1-10 nM) in the presence and absence of 10^{-6}M unlabelled DAla^{6}EA.

3.6.2.10 *Effect of Extreme Temperature*: Rabbit pituitary membrane preparations were heated to 45-55°C for 10 minutes prior to use in the binding assay. Membrane preparations maintained at 4°C served as a control.

3.6.2.11 *Effect of Ions*: The effect of ions on ^{125}I-DAla^{6}EA binding was studied by the addition of $CaCl_2$ (0.1,1.0,10 mM), $MgCl_2$ (0.1,1.0,10 mM) and NaCl (30,150,300 mM) to the assay buffer. A fixed ligand concentration (approximately 0.4 nM) was added to pituitary membrane preparations with and without 10^{-6}M unlabelled DAla^{6}EA.

3.6.2.12 *Effect of Freezing*: Rabbit pituitaries were quickly removed from adult animals and either assayed immediately or frozen (quick frozen in liquid nitrogen and stored at -70°C). Frozen samples were analyzed for ^{125}I-DAla^{6}EA binding 2 weeks, 3 weeks, 4 weeks or 12 weeks following freezing. GnRH binding in individual pituitaries was determined by measuring ^{125}I-DAla^{6}EA binding at a fixed ligand concentration (approximately 0.4 nM). In addition, the K_d and B_{max} of ^{125}I-DAla^{6}EA binding were measured using a pool of membrane preparation (n=6) and increasing concentrations of labelled DAla^{6}EA (0.1-10 nM). NSB was determined by the addition of 10^{-6}M unlabelled DAla^{6}EA.

3.6.2.13 *Tracer Degradation*: A fixed concentration of ligand was incubated with pituitary membrane preparations. Following 60 or 120 minutes of incubation at 4°C, the tubes were centrifuged (15 min at 11,000 x g). The resulting supernatant was then applied to a fresh aliquot of membrane preparation, incubated for 60 minutes at 4°C and centrifuged. These second pellets were counted in addition to those pellets obtained from the initial incubation. The extent of degradation was determined by

the ability of the unbound tracer in the supernatant to bind to the second fresh aliquot of membrane preparation.

3.6.2.14 *Kinetics of ^{125}I-DAla^{6}EA Binding*: The association rate of ^{125}I-DAla^{6}EA binding was examined by incubation of pituitary membrane preparations with approximately 0.4 nM labelled ^{125}I-DAla^{6}EA for 3-90 minutes. To determine the dissociation rate of the labelled ligand, an excess (10^{-6}M) of unlabelled DAla^{6}EA was added following 60 minutes of incubation at 4°C. The reaction was allowed to continue for various time intervals (3-60 min). To separate bound from free ligand, the preparations were poured onto a Whatman GF/C filter (soaked overnight in 0.3% PEI), then washed 3 times with 4 ml ice-cold incubation buffer using rapid vacuum filtration. Radioactivity on filters was counted by γ-spectroscopy.

3.6.3 DOWN REGULATION OF ^{125}I-DALA^{6}EA BINDING

Adult female NZW rabbits (163 days of age, 3.0-5.0 kg) were injected s.c. with 100 ng/kg DAla^{6}EA (n=7), 100 ng/kg DpGlu^1 (n=7) or saline (n=6) every 12 hours (8 am:8 pm) for 4 days. Animals were sacrificed by euthanasia (65 mg sodium pentobarbital per ml; 1 ml/kg body weight administered into superficial ear vein) at least 12 hours post-final injection. Peripheral blood was collected in heparinized tubes and centrifuged for 15 minutes at 600 x g. Serum was frozen at -20°C until assayed for LH. Anterior pituitaries were rapidly removed, frozen in liquid nitrogen

and stored at -70°C until assayed for ^{125}I-DAla^{6}EA binding.

GnRH binding in individual pituitaries was determined by measuring ^{125}I-DAla^{6}EA binding at a fixed labelled DAla^{6}EA concentration and standard assay conditions. Measurements for all treatment groups were carried out in the same assay to avoid interassay variation.

To determine ^{125}I-DAla^{6}EA apparent dissociation constant (K_d) and maximal binding capacity (B_{max}), 100 μl aliquots from a pool of membrane preparations (n=6) for each treatment group were incubated with increasing concentrations of labelled DAla^{6}EA (0.1-5 nM) with or without the addition of 10^{-6}M unlabelled DAla^{6}EA. Standard assay procedures were followed.

3.6.4 ONTOGENY OF ^{125}I-DALA^{6}EA BINDING IN THE FEMALE RABBIT

Female rabbits of defined ages were allowed to acclimatize to living conditions for 2 days after arrival. Animals were sacrificed at 25, 40, 50, 65, 75, 85 and 120 days of age. Sodium pentobarbital (2 ml per kg body weight) was administered intraperitoneally to animals aged 25 and 40 days. The older animals were euthanized by injection (65 mg sodium pentobarbital per ml; 1 ml per kg body weight) into a superficial ear vein. Anterior pituitaries were rapidly removed, frozen in liquid nitrogen and stored at -70°C until assayed for ^{125}I-DAla^{6}EA binding. The sex of animals was confirmed by the presence of ovaries. Serum was stored at -20°C until assayed for LH.

GnRH binding in individual pituitaries was determined by measuring ^{125}I-DAla^{6}EA binding at a fixed radioligand concentration (approximately 0.4 nM) and standard assay conditions. All age groups were included in a single assay which was carried out in triplicate (3 total, 3 NSB). The assay was conducted 3 times with separate pituitary membrane preparations.

3.6.5 BINDING CONSTANTS OF ^{125}I-DALA^{6}EA BINDING TO FEMALE RABBIT PITUITARIES (40, 75 AND 120 DAY)

Pituitaries from female NZW rabbits were collected at defined ages after birth (40 days, n=48; 75 days, n=21; and 120 days, n=20), frozen in liquid nitrogen and stored at -70°C until assayed. To determine ^{125}I-DAla^{6}EA apparent dissociation constant (K_d) and maximal binding capacity (B_{max}), increasing concentrations of labelled ligand (0.1-10 nM) were added to pooled pituitary membrane preparations for each age group in the presence and absence of 10^{-6}M unlabelled DAla^{6}EA.

3.7 IN VIVO LH RESPONSE TO DALA^{6}EA

Female rabbits aged 40 days, 75 days and 120 days (n=5-7 per group) were injected s.c. with 1 ml of DAla^{6}EA at various concentrations in 0.09% NaCl. Three dosages of DAla^{6}EA (10 ng, 100 ng and 1 μg per kg body weight) were administered to separate groups of animals for all 3 age categories. Following administration of DAla^{6}EA, blood samples were taken at 15, 30, 45 and 120 minutes. Animals were

bled via the middle ear artery (1-2 ml blood samples) with gauge 22 needles coated with heparin (1000 i.u./ml). Baseline blood samples were taken 2 days prior to DAla^{6}EA injection to minimize the stress of multiple sampling. Animals were placed in a metal rabbit restrainer during blood sampling only. Samples were centrifuged and serum was stored at -20°C until assayed for LH.

3.8 IN VITRO LH RESPONSE TO GnRH AND GnRH ANALOGS

Pituitary glands from adult female NZW rabbits (2.5-3.0 kg) were collected into Ham's F10 medium containing 10 percent human serum albumin. After collection, anterior pituitaries were sliced at 350 μm using a McElwain Tissue Chopper. Tissue slices were carefully tweezed apart under a dissecting microscope using fine tweezers. Individual tissue slices were then placed into separate wells of tissue culture plates (Nunclon 24-well) containing 500 μl of medium. Tissue slices were incubated at 37°C in a water-saturated atmosphere of 95% O_2/5% CO_2 for 2 hours to allow stabilization of LH release. Following this 2 hour preincubation period, a 100 μl sample was collected from each well to determine baseline LH. Media was replaced in all wells. Experimental wells received GnRH analogs (Factrel, DLeu^{6}EA, DAla^{6}EA and native GnRH) in increasing dosages (10^{-11}M to 10^{-7}M). Wells receiving media only served as controls. At the end of 4 hours, samples for LH released were collected by removing a 100 μl sample of medium. Samples were frozen at -20°C until assayed for LH. The weight of each tissue slice was recorded

at the end of the experiment. LH release at each GnRH analog concentration was determined in triplicate. The ED_{50} for each dose-response curve was calculated using a computerized curve fitting programme. Each experiment was conducted 3 times with separate pituitary tissues.

3.9 ANALYSIS OF RABBIT SERUM LUTEINIZING HORMONE

LH was measured by radioimmunoassay using previously established techniques (Moor and YoungLai, 1975). The LH standard used was WP360A (Dr. A.F. Parlow), 1 ng of which is equivalent to 30 pg pure rabbit pituitary LH (EX130GB, Dr. L.E. Reichert, Jr.). Guinea pig anti-rabbit LH 6F GPāLH (Dr. R. Scaramuzzi) was used as primary antibody. Purified ovine LH was used for iodination (LER-1056-C2)(Dr. L.E. Reichert, Jr.). All LH results were expressed in terms of pure pituitary LH standard. The sensitivity of the assay was 42 pg. The intra- and inter-assay coefficients of variation were 5.4% and 12.7% respectively. (See Appendices D and E for iodination and RIA protocols)

3.10 PRESENTATION OF RESULTS AND STATISTICAL ANALYSIS

Data presented are the means ± standard error of the means (SEMs). Results for ^{125}I-DAla^{6}EA binding to rabbit anterior pituitary membrane preparations are presented as means ± SEMs of fmol/mg protein. A computerized nonlinear least-squares curve-fitting programme (BDATA) was used for analysis of saturation

experiments to estimate apparent dissociation constants (K_d) and maximal binding capacity (B_{max}). IC_{50}'s for competition curves were also determined using a non-linear curve-fitting programme (CDATA). Both programmes allowed for data to be fitted separately for a single class and two classes of binding sites. Curves were analyzed using statistical comparison (F-test) of the residual squares generated by curve fitting.

Statistical analysis was performed using analysis of variance (ANOVA) in conjuction with Scheffés post-hoc test. Analysis was conducted using BMDP (Biomedical Computer Programmes). Level of significance was chosen as $p<0.05$.

CHAPTER FOUR

RESULTS

4.1 IODINATION OF DALA^{6}EA

A typical elution profile from the Sephadex G-25 column is shown in Figure 2. ^{125}I-labelled DAla^{6}EA elutes in the second peak, following free ^{125}I. This iodination profile is a result of the so-called aromatic adsorption effect *i.e.* retardation of the tyrosine molecule at a low pH of eluant (see Regoeczi, 1984). The four fractions of the DAla^{6}EA labelled peak were routinely used in the binding assay. The specific activity of ^{125}I-labelled DAla^{6}EA ranged from 875 to 1250 μCi/μg (see Appendix G).

The iodinated DAla^{6}EA was stored at acid pH (approximately 4.5) at 4°C and lasted 3-4 weeks. Prior to use in the binding assay an aliquot of the label was removed and blown down under compressed nitrogen gas. The activity of the label as well as batch to batch variation were determined by saturation binding to female rat pituitary membrane preparations.

4.2 PITUITARY MEMBRANE PREPARATION

Crude membrane preparation of rabbit pituitaries is depicted in Figure 3. Table 2 shows a comparison of the amount of specific binding in various steps of the membrane preparation. The 14,000 x g fraction (pellet 2) contained the largest amount of ^{125}I-DAla^{6}EA binding (71.90±7.29 fmol/mg protein) and was significantly higher ($p<0.05$) than pellet 1 (600 x g fraction)(38.40±11.96 fmol/mg protein). ^{125}I-DAla^{6}EA binding to P_2 was higher but not significantly different from the binding to the whole homogenate (61.26±5.50 fmol/mg protein) or the 100,000 x g fraction

FIGURE 2: A typical elution profile of ^{125}I-labelled DAla^{6}EA (1.5 ml fractions) from a Sephadex G-25 fine column eluted with 0.1N acetic acid - 0.25% BSA. Fractions 23-26 were pooled and used for the radioligand binding assay.

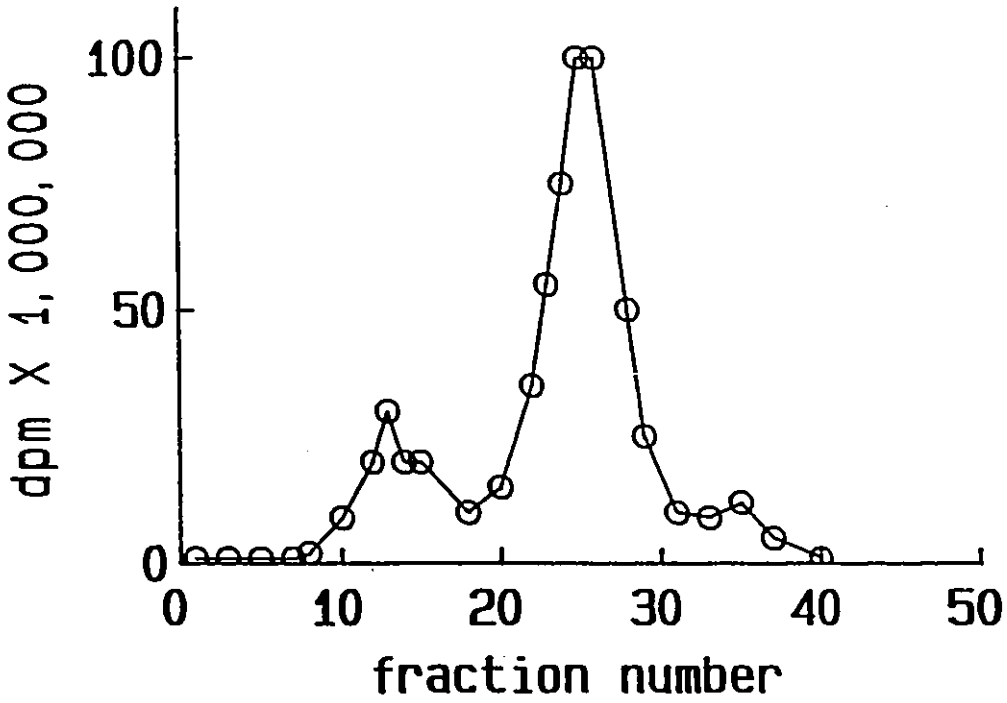

100
50
0
dpm X 1,000,000
0
10
20
30
40
50
fraction number

FIGURE 3: Membrane preparation of rabbit anterior pituitaries for use in the ^{125}I-DAla^{6}EA binding assay. The 14,000 x g pellet (P_2) was the standard preparation used in the binding assay.

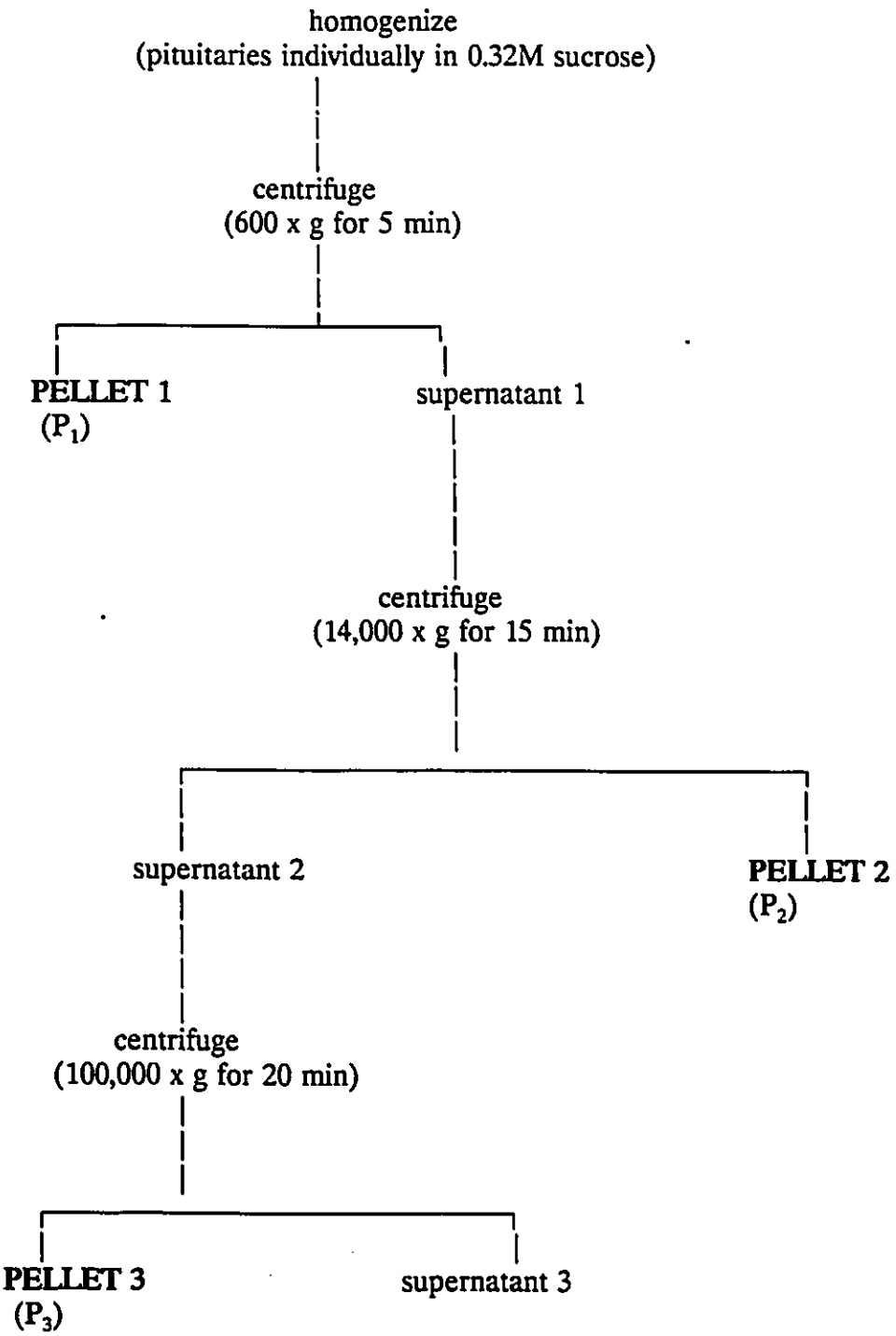

homogenize
(pituitaries individually in 0.32M sucrose)
centrifuge
(600 x g for 5 min)
PELLET 1
(P_1)
supernatant 1
centrifuge
(14,000 x g for 15 min)
supernatant 2
PELLET 2
(P_2)
centrifuge
(100,000 x g for 20 min)
PELLET 3
(P_3)
supernatant 3

TABLE 2: Membrane preparation of anterior pituitaries of adult rabbits for use in the ^{125}I-DAla^{6}EA binding assay

Fraction	Specific Binding (fmoles/mg protein)
whole homogenate	61.28 ± 5.50 (4)
P_1 fraction (600 x g)	38.40 ± 11.96 (5)
P_2 fraction (14,000 x g)	71.90 ± 7.29 (6)
P_3 fraction (100,000 x g)	54.53 ± 16.71 (3)

Values represent the means ± SEMs.
Number of experiments in parentheses, 3 observations per experiment (3 total, 3 NSB).
P_2 fraction was used for all subsequent assays.

(54.53 ± 16.71 fmol/mg protein)(pellet 3).

The 14,000 x g fraction (P_2) was the standard preparation used in the ^{125}I-DAla^{6}EA binding assay. This crude membrane preparation has been used in radioligand binding assays by several investigators to measure pituitary GnRH binding site concentration and/or affinity in the rat (Duncan et al, 1983; Dalkin et al, 1981) and rabbit (Limonta et al, 1986; Thorson et al, 1985). Rapid and dramatic changes in GnRH binding sites have been shown in animals of various endocrine states using this membrane preparation (see Clayton, 1989). In order to compare the present results with previous GnRH analog binding data, a similar crude membrane preparation was used. Furthermore, the highest amount of ^{125}I-DAla^{6}EA specific binding was evident in the 14,000 x g pellet of the rabbit pituitary membrane preparation. Thus, GnRH binding sites in the crude membrane fraction (P_2) were of primary interest for the present study.

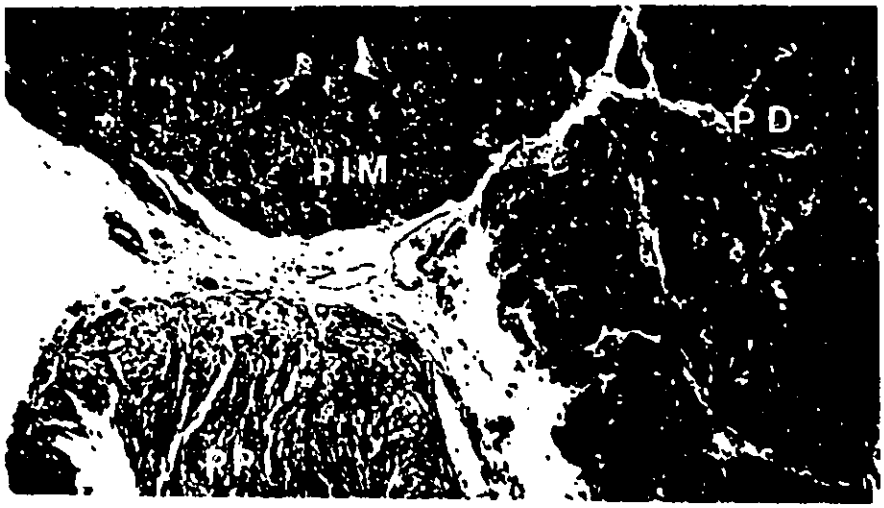
4.3 HISTOLOGY OF RABBIT PITUITARIES

Hematoxylin and eosin staining of rabbit pituitaries were carried out to ensure proper dissection of the anterior pituitary for use in the radioligand binding assay. Figure 4A shows the posterior (neurohypophysis) and anterior (adenohypophysis) lobes of the rabbit pituitary. The cells of the posterior pituitary, the pituicytes, which resemble the neuroglia cells of the central nervous system are evident in Figure 4B. Figure 4C demonstrates the highly vascular anterior pituitary containing the cell types

FIGURE 4A: The anterior (adenohypophysis) and posterior (neurohypophysis) pituitary (PP) of the rabbit stained with hematoxylin and eosin. The anterior pituitary can be divided into the pars distalis (PD) and pars intermedius (PIM). (x45)

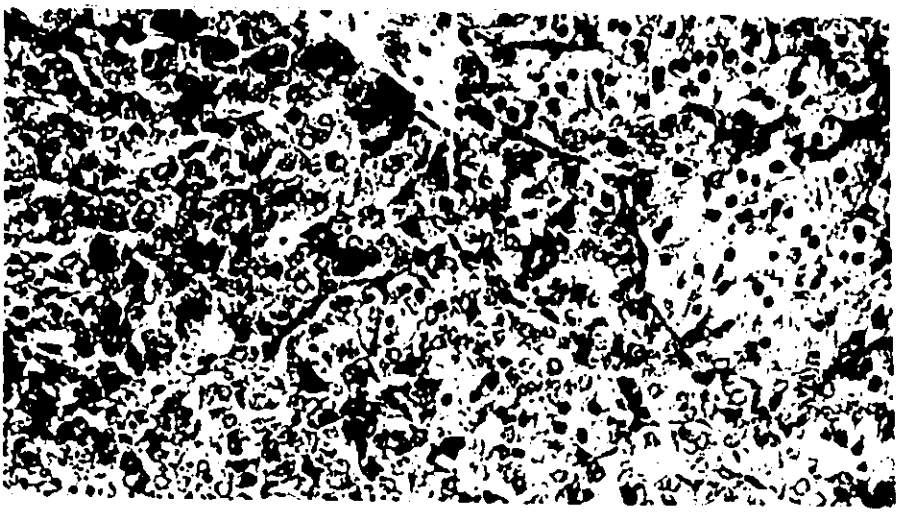
FIGURE 4B: The neurohypophysis of the rabbit. (x100)

FIGURE 4C: Representative area of the anterior pituitary (pars distalis). (x45)



4A

4B



4C

chromophobes, acidophils, and basophils.

4.4 GnRH BINDING ASSAY

4.4.1 STANDARD GnRH BINDING ASSAY CONDITIONS

4.4.1.1 *Blanks*

Optimal conditions for minimizing background counts (blanks) were determined by variation in the number of washes, volume of wash buffer and precoating of tubes with BSA. Conditions chosen for reducing background counts were precoating polypropylene microcentrifuge tubes overnight with 2% BSA (Figure 5), washing with 400 μl buffer (Figure 6) and 3 washes with assay buffer (Figure 7). Centrifugation appeared to be the optimal method of separating bound from free radioligand when compared to filtration (Figure 8). Less than one percent of the total radioligand added bound to the tube.

4.4.1.2 *Optimal Incubation Conditions*

The number of washes, volume of wash buffer and amount of BSA in the incubation medium was varied to determine the optimal incubation conditions of specific ^{125}I-DAla^{6}EA binding to rabbit pituitary membrane preparations (Figures 9 and 10). From these experiments, 3 washes with 400 μl assay buffer (50 mM Tris HCl, pH 7.4) was chosen as optimal conditions to reduce NSB. The incubation media contained 0.1% BSA since specific binding was increased 13.01 ± 1.04 and 10.04 ±

FIGURE 5: Effect of precoating incubation tubes overnight with bovine serum albumin (BSA) on background counts. Values are the means ± SEMs of 3 experiments, each carried out in triplicate (3 total, 3 NSB) according to incubation conditions outlined in Methods. NSB was measured in the presence of 10^{-6}M unlabelled DAla^{6}EA. Numbers represent percent of ^{125}I-DAla^{6}EA added (0.5-1.0 nM) bound to tubes.

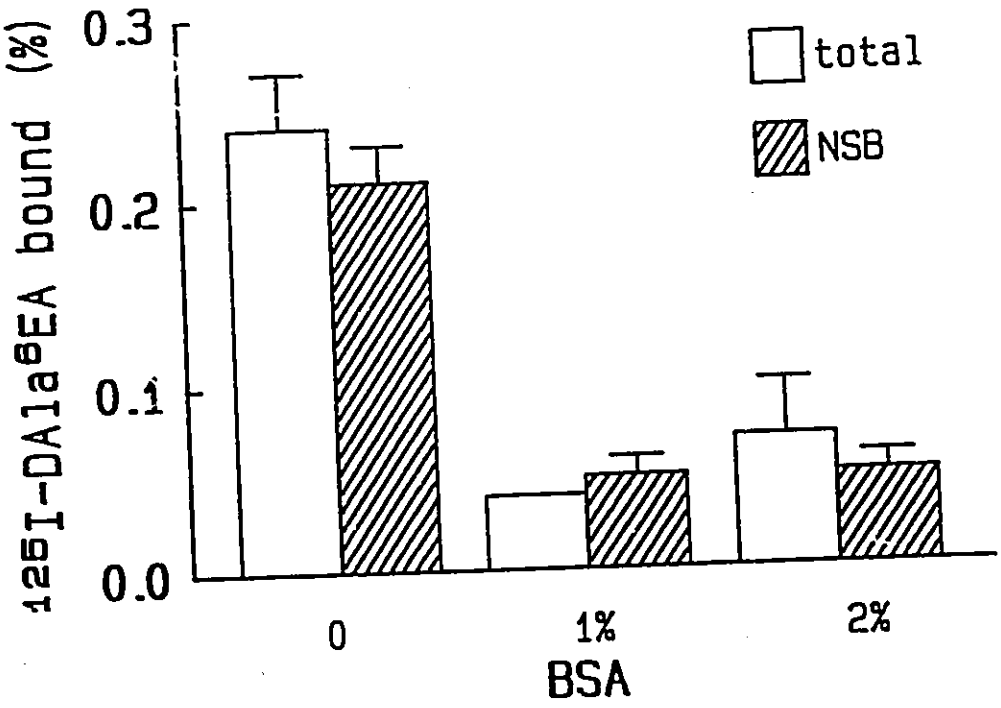

0.3
0.2
0.1
0.0
125I-DA1aβEA bound (%)
total
NSB
0
1%
2%
BSA

FIGURE 6: Effect of volume of wash buffer on background blanks. Tubes were washed 3 times with Tris-HCl buffer (50 mM, pH 7.4) at specified volumes. Values are the means ± SEMs of 2 experiments, each carried out in triplicate (3 total, 3 NSB) according to incubation conditions outlined in Methods. Numbers represent percentage of ^{125}I-DAla^{6}EA added (0.5-1.0 nM) bound to tubes.

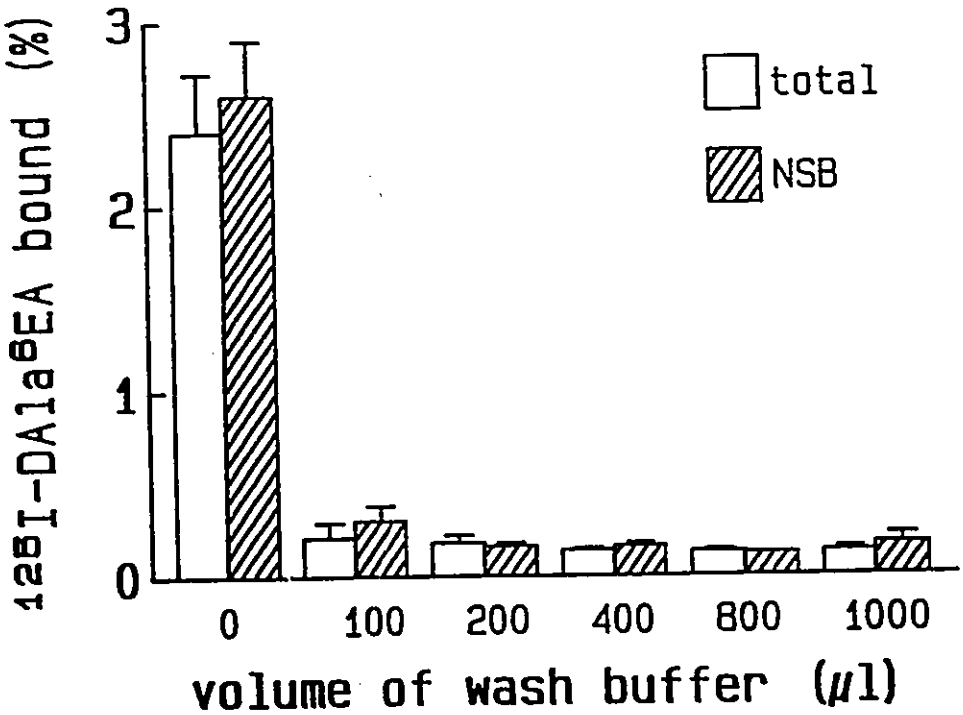

125I-DAla8EA bound (%)
3
2
1
0
total
NSB
0
100
200
400
800
1000
volume of wash buffer (μl)

FIGURE 7: Effect of number of washes on blanks. Tubes were washed with 400 μl Tris-HCl buffer (50 mM, pH 7.4) following 60 minutes of incubation at 4°C and centrifugation (15 min @ 11,000 x g). Values are means ± SEMs of 2 experiments, each carried out in triplicate (3 total, 3 NSB). NSB was measured in the presence of 10^{-6}M unlabelled DAla^{6}EA. Numbers represent the percentage of ^{125}I-DAla^{6}EA added (0.5-1.0 nM) bound to tubes.

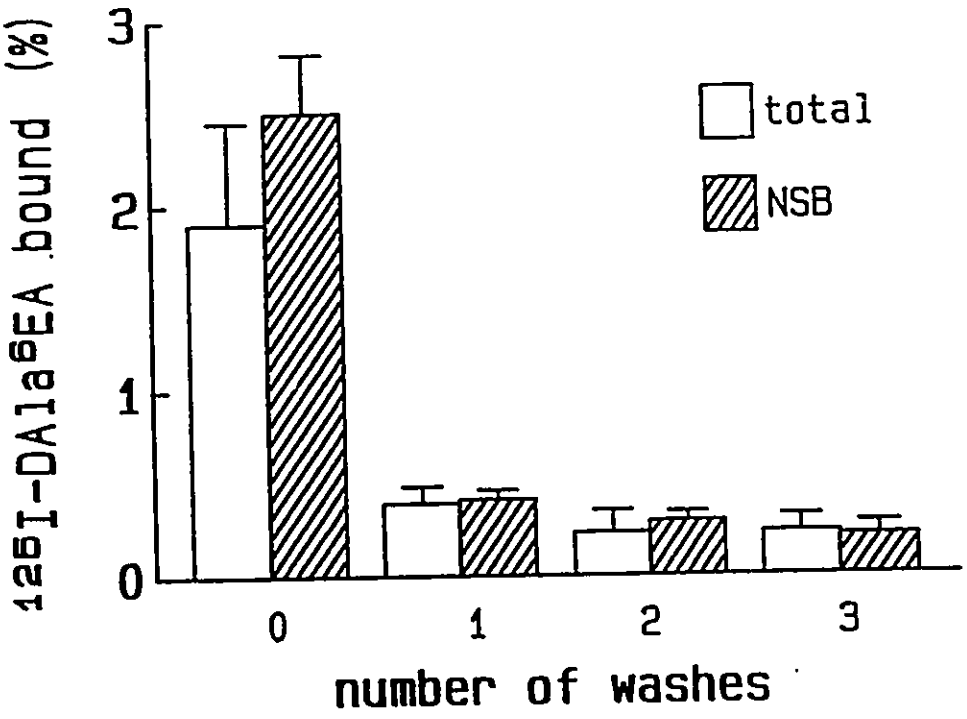

125I-DAla6EA bound (%)
3
2
1
0
total
NSB
0
1
2
3
number of washes

FIGURE 8: Blanks obtained using filtration or centrifugation methods to separate bound and free ^{125}I-DAla^{6}EA. Values are the means ± SEMs of 3 experiments, each carried out in triplicate (3 total, 3 NSB) according to incubation conditions outlined in Methods. NSB was determined by parallel incubations carried out in the presence of 10^{-6}M unlabelled DAla^{6}EA. C = centrifugation; F = filtration; BSA = bovine serum albumin; PEI = polyethyleneimine. Numbers represent percentage of ^{125}I-DAla^{6}EA added (0.5-1.0 nM) bound to tubes.

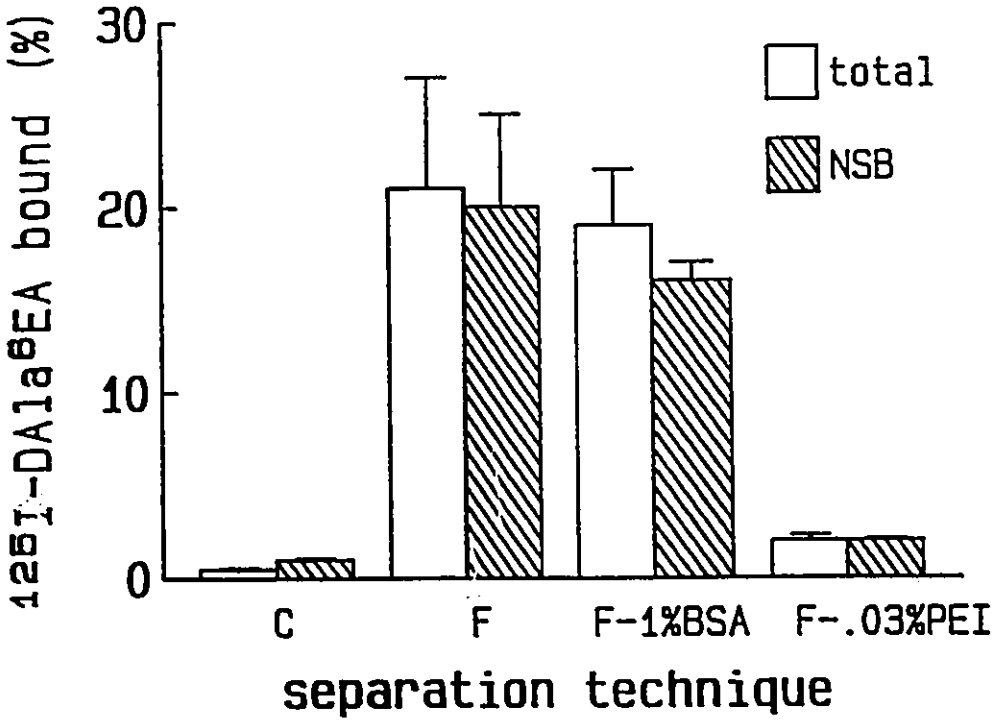

30
20
10
0
125I-DA1aBEA bound (%)
total
NSB
C
F
F-1%BSA
F-.03%PEI
separation technique

FIGURE 9: Effect of number of washes with assay buffer (50 mM Tris, pH 7.4) on ^{125}I-DAla^{6}EA (0.5-1.0 nM) binding to rabbit anterior pituitary membrane preparations. Following 60 minutes of incubation at 4°C and centrifugation (15 min @ 11,000 x g), the remaining pellets were washed with 400 µl buffer for the specified number of times. NSB was obtained by parallel incubations carried out in the presence of 10^{-6}M unlabelled DAla^{6}EA. Specific binding = total binding minus non specific binding. Values are the means of 2 experiments, each carried out in triplicate (3 total, 3 NSB).

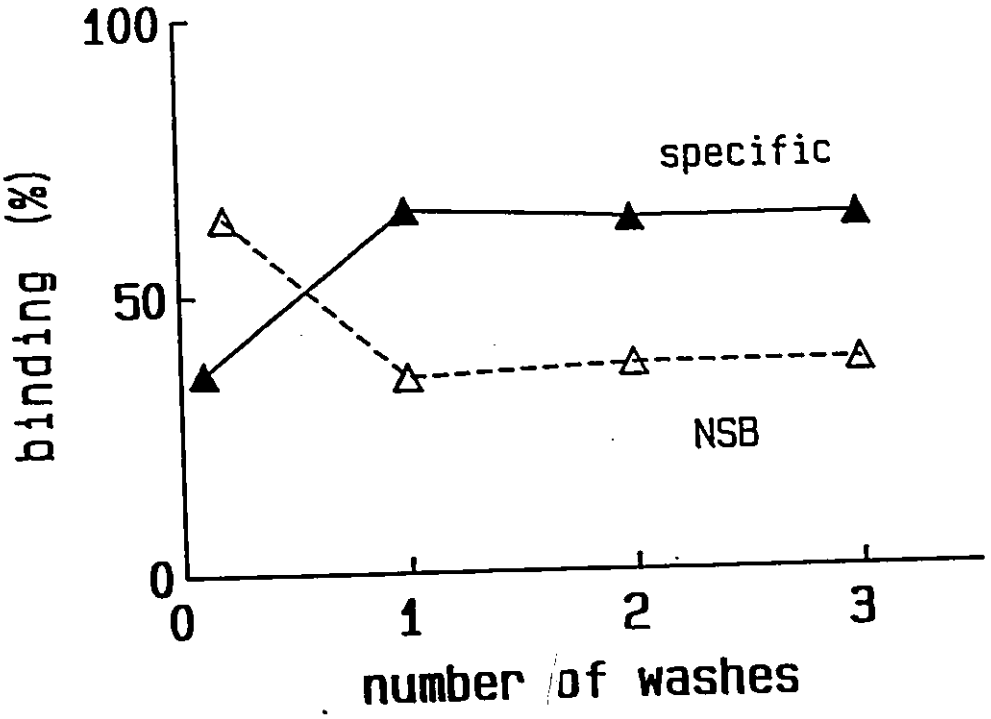
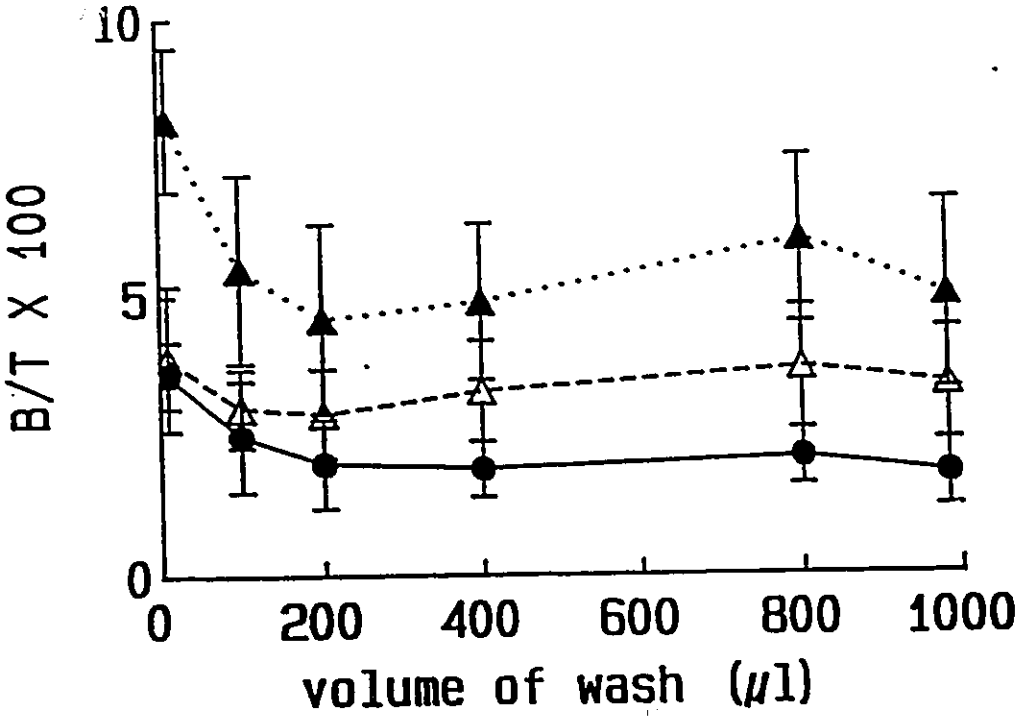

100
50
0
binding (%)
specific
NSB
0
1
2
3
number of washes

FIGURE 10: Effect of wash volume on ^{125}I-DAla^{6}EA (0.5-1.0 nM) binding to rabbit membrane preparations. Following 60 minutes of incubation and centrifugation (15 min @ 11,000 x g), the remaining pellets were washed 3 times with the specified volume of wash buffer (50 mM Tris, pH 7.4). NSB was determined in the presence of 10^{-6}M unlabelled DAla^{6}EA. Values are the means ± SEMs of 3 experiments. ▵ = specific binding; ▲ = total binding; ● = non specific binding.


10
5
0
B/T X 100
0
200
400
600
800
1000
volume of wash (µl)

6.94 percent when compared to tubes that contained 1% BSA or no BSA, respectively. Under the optimal assay conditions, intra-assay variations were 4.79, 10.36 and 9.51 percent for total, non-specific and specific binding (Table 3).

4.4.2 CHARACTERIZATION OF ^{125}I-DALA^{6}EA BINDING

4.4.2.1 *Tissue Linearity*

The correlation between tissue concentration and ^{125}I-DAla^{6}EA binding over the range of 20-240 μg protein per tube is shown in Figure 11. ^{125}I-DAla^{6}EA binding was found to be a function of rabbit pituitary membrane concentration with a positive linear correlation for total (.997), nonspecific (.984) and specific (.996) binding.

4.4.2.2 *Temperature Dependence*

The effect of temperature of incubation on specific ^{125}I-DAla^{6}EA binding is shown in Figure 12. Binding was significantly reduced at 37°C and 30°C when compared to 4°C ($p<0.05$). At room temperature (22-24°C) specific binding was reduced by 34.40 percent when compared to 4°C. The optimal temperature for ^{125}I-DAla^{6}EA binding to rabbit anterior pituitary membrane preparation of 4°C was used as a standard experimental temperature for subsequent assays.

TABLE 3: Intra-assay variability of ^{125}I-DAla^{6}EA binding to rabbit anterior pituitary membrane preparations

	Binding (dpm)		
	mean	SD	% variability
Total	2712	130	4.79
NSB	820	85	10.36
Specific	1893	180	9.51

Values derived from 12 determinations.
Variability = SD/mean X 100
Approximately 0.4 nM ^{125}I-DAla^{6}EA was added per tube.
Standard incubation conditions were met.

FIGURE 11: Equilibrium ^{125}I-DAla^{6}EA (approximately 0.4 nM) binding to increasing concentrations of rabbit pituitary crude membrane preparation (20 μg - 240 μg protein per tube). NSB was obtained by parallel incubations carried out in the presence of 10^{-6}M unlabelled DAla^{6}EA. Specific binding = total binding minus NSB. Values are means ± SEMs of triplicate determinations. Correlation coefficients obtained were .997, .984 and .996 for total, NSB and specific binding, respectively. Results were repeated twice.

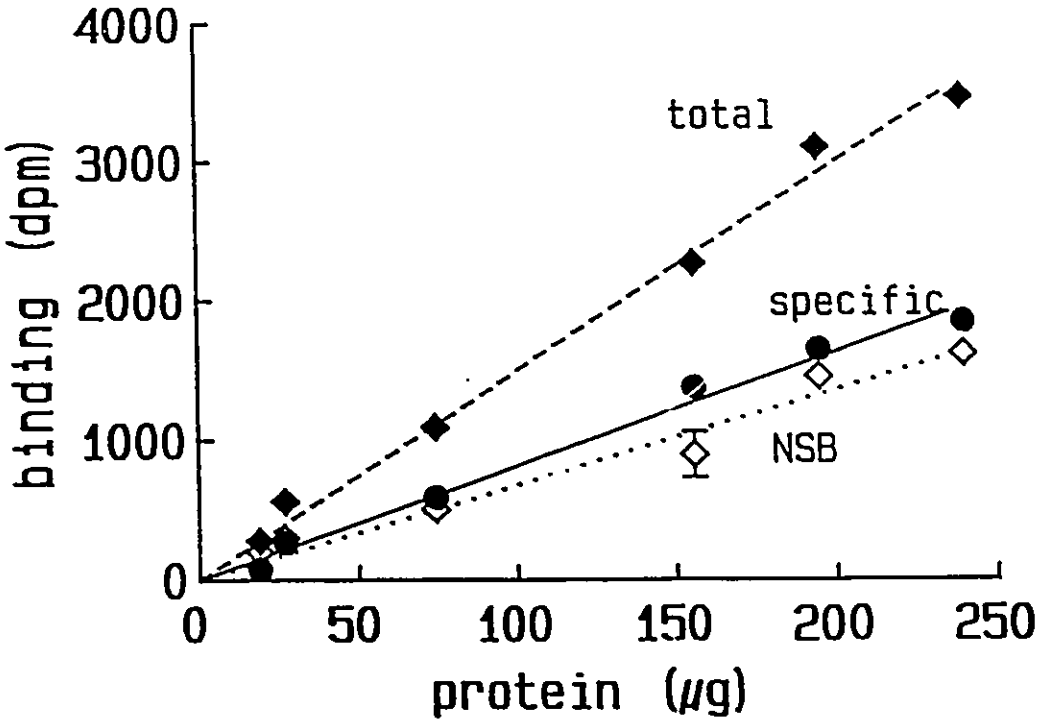
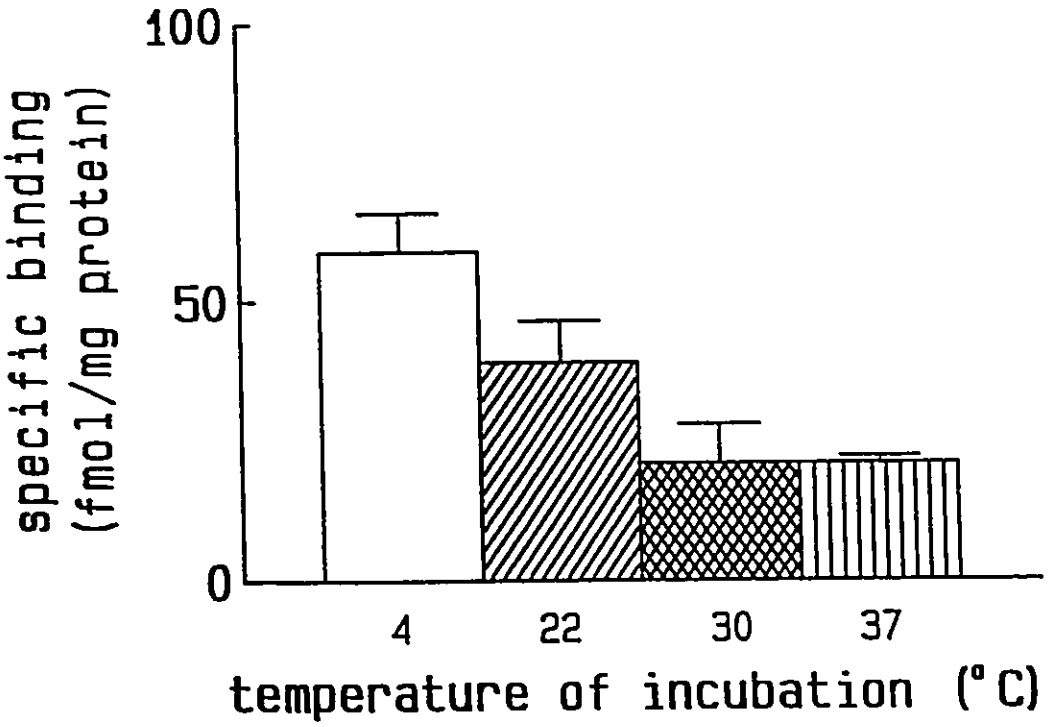

4000
3000
2000
1000
0
binding (dpm)
total
specific
NSB
0
50
100
150
200
250
protein (µg)

FIGURE 12: Temperature dependence of ^{125}I-DAla^{6}EA (0.4 nM) binding to adult rabbit anterior pituitary membrane preparations (14,000 x g, 60-110 μg protein per tube). Data show specific binding following 60 minutes of incubation at temperatures 4°C, 22-24°C, 30°C and 37°C. NSB was assessed in the presence of 10^{-6}M unlabelled DAla^{6}EA. Values are means ± SEMs of 4 experiments, each carried out in triplicate (3 total, 3 NSB). Specific binding was significantly higher at 4° C when compared to 22°C, 30°C and 37°C ($p<0.05$).


100
50
0
specific binding
(fmol/mg protein)
4
22
30
37
temperature of incubation (°C)

4.4.2.3 *Time Course and Temperature of Incubation*

The time course of ^{125}I-DAla^{6}EA binding shown in Figure 13 demonstrates that at 4°C equilibrium binding was reached at 45-60 minutes and specific binding remained stable for the following 60 minutes. In contrast, increasing the temperature of incubation to 30°C significantly reduced specific ^{125}I-DAla^{6}EA binding ($p<0.05$). An incubation period of 60 minutes at 4°C was used as a standard experimental condition for all subsequent assays.

4.4.2.4 *Effect of Bacitracin, DTT and Soybean Trypsin Inhibitor on ^{125}I-DAla^{6}EA Binding*

Table 4 shows the effect of the addition of bacitracin (100 μg/ml), DTT (0.1M) or soybean trypsin inhibitor (1 mg/ml) to the incubation medium on ^{125}I-DAla^{6}EA binding at 4°C. Compared to control (buffer only), DTT, soybean trypsin inhibitor and bacitracin did not affect ^{125}I-DAla^{6}EA binding to rabbit pituitary membrane preparation ($p>0.05$). Thus, these agents were not included in the standard ^{125}I-DAla^{5}EA binding assay conditions.

4.4.2.5 *Maximal Bindability*

Not all of the radioiodinated ligands added are able to bind, possibly because some of the labelled hormone has been "damaged or altered" in the process of iodination or separation (Clayton, 1985). The maximal bindable fraction of labelled

FIGURE 13: Time and temperature dependence of ^{125}I-DAla^{6}EA (0.4 nM) binding to rabbit anterior pituitary membrane preparations. Data show binding corrected for NSB measured in the presence of 10^{-6}M unlabelled DAla^{6}EA. Values are means ± SEMs of 6 observations (2 experiments, each carried out in triplicate).

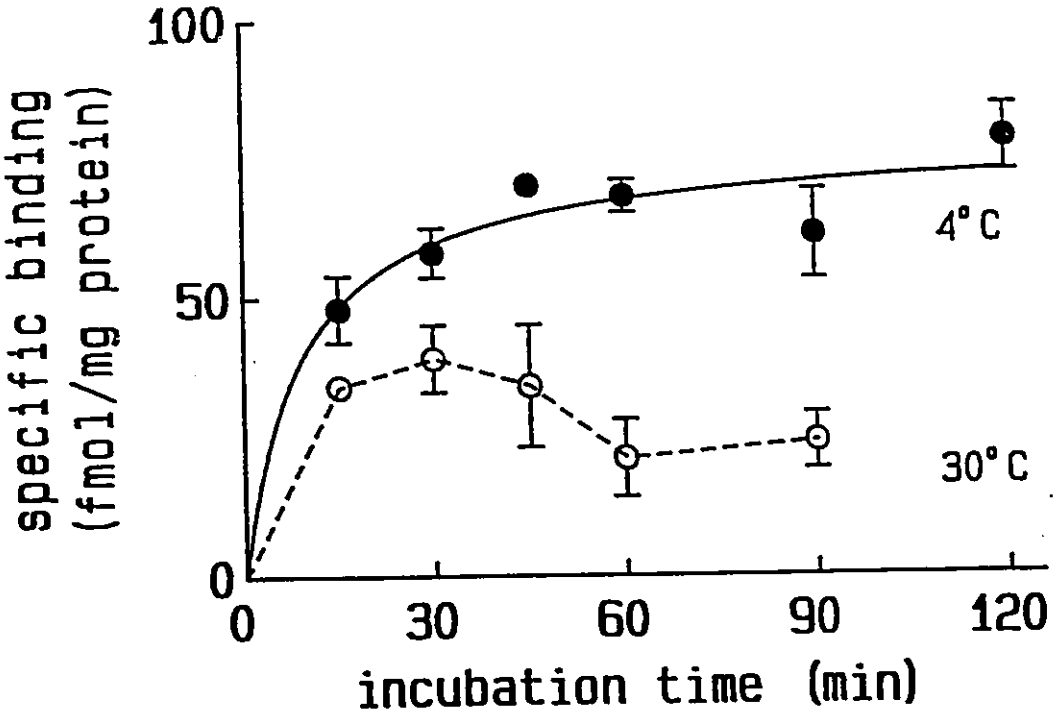

100
50
0
specific binding
(fmol/mg protein)
4° C
30° C
0
30
60
90
120
incubation time (min)

TABLE 4: Effect of the addition of various agents to the incubation medium on specific binding of ^{125}I-DAla^{6}EA to rabbit anterior pituitary membrane

Agent	Specific Binding (% of controls)
bacitracin (100 μg/ml)	87.2 ± 4.6
dithiothreitol (0.1 M)	89.6 ± 3.3
soybean trypsin inhibitor (1 mg/ml)	93.5 ± 5.0

Data represent the means ± SEMs of 3 assays, each carried out in triplicate (3 total, 3 NSB). Approximately 0.4 nM ^{125}I-DAla^{6}EA added per tube. Controls = incubation medium only (50 mM Tris-HCl, 0.1% BSA). Specific binding was not significantly different from controls ($p > 0.05$).

^{125}I-DAla^{6}EA was assessed by incubation of a small concentration of labelled DAla^{6}EA with increasing concentrations of membrane preparation. Figure 14 illustrates the curvilinear relationship between tissue concentration and amount of labelled DAla^{6}EA that will bind. The maximal bindable fraction of ^{125}I-DAla^{6}EA routinely achieved (as indicated by the plateau) was approximately 70 percent.

4.4.2.6 *Competition Curves*

The specificity of ^{125}I-DAla^{6}EA binding to rabbit anterior pituitary membrane preparations was confirmed by displacement experiments with increasing concentrations of unlabelled GnRH and GnRH analogs (Figure 15). The IC_{50}s for GnRH analogs DAla^{6}EA, DLeu^{6}EA and Gonadorelin Hydrochloride (Factrel) were 1.36±0.11 nM, 0.69±0.13 nM and 1.58±0.52 nM, respectively (Table 5). The GnRH antagonist DpGlu^1 produced an IC_{50} of 2.04±0.86 nM (Table 5). Native GnRH was inactive up to 10^{-6}M with a IC_{50} of 10.80±3.00 μM. Peptides unrelated to GnRH (arginine vasopressin, oxytocin, LH, FSH and TSH) showed minimal displacement of labelled DAla^{6}EA even at high concentrations (10^{-5}M, 10^{-6}M). The calculated K_is from competition experiments shown in Table 5 for DAla^{6}EA, DLeu^{6}EA, Factrel, native GnRH and DpGlu^1 were 1.12±0.01 nM, 0.56±0.11 nM, 1.52±0.47 nM, 8.80±2.7 μM and 1.74±0.77 nM, respectively.

FIGURE 14: Determination of maximal bindable fraction of ^{125}I-DAla^{6}EA. A low concentration (2.5 x 10^{-11}M) of radiolabelled DAla^{6}EA was incubated with increasing amounts of rabbit pituitary membrane preparation. A plateau was reached when 70 percent of the added tracer was bound by excess receptor. Results are means ± SEMs of 3 observations, data collected from 3 separate experiments.

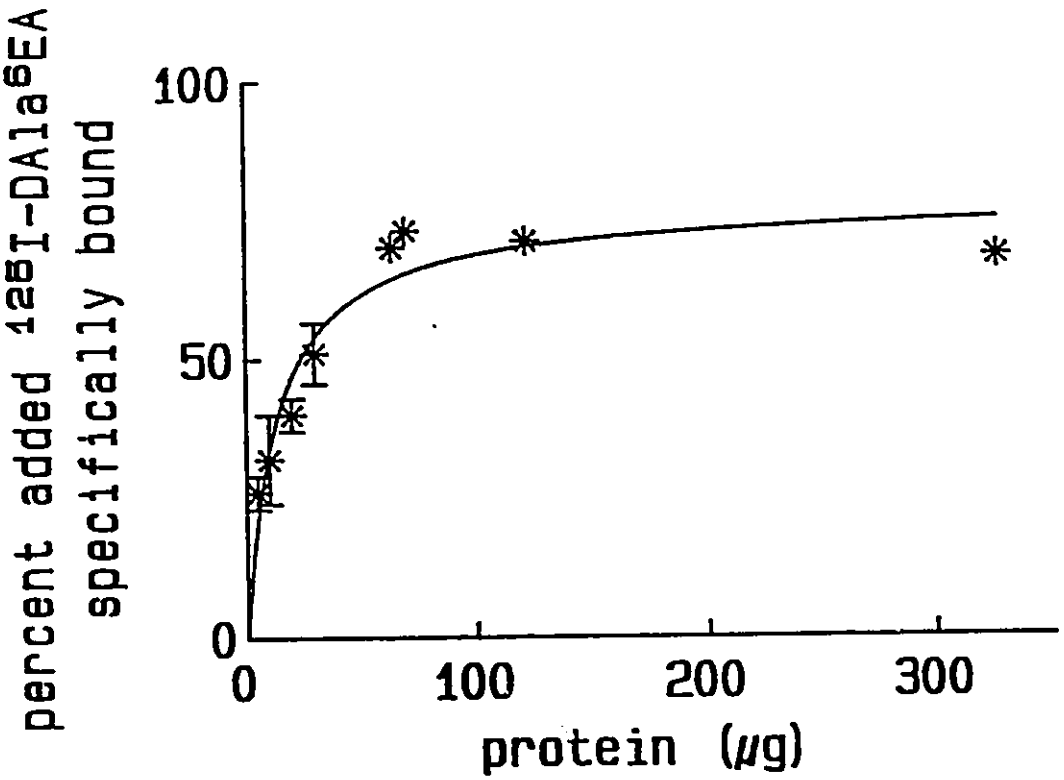

percent added ^{125}I-DAla^{6}EA specifically bound
100
50
0
0
100
200
300
protein (μg)

FIGURE 15: Competition curves for ^{125}I-DAla^{6}EA (0.4 nM) binding rabbit anterior pituitary membrane preparations. Data shown are representative experiments. For each experiment triplicate determinations were made at each point (60-90 μg protein per tube). Values represent the percentage of binding corrected for NSB. □ = arginine vasopressin; ○ = des-Gly^{10}[DAla^{6}] GnRH ethylamide; + luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone; ▵ = native gonadotropin releasing hormone; ▲ = oxytocin; ● = des-Gly^{10}[DLeu^{6}]GnRH ethylamide; ◇ = prostaglandin E_2; ◆ = prostaglandin $F_{2\alpha}$; ■ = gonadorelin hydrochloride.

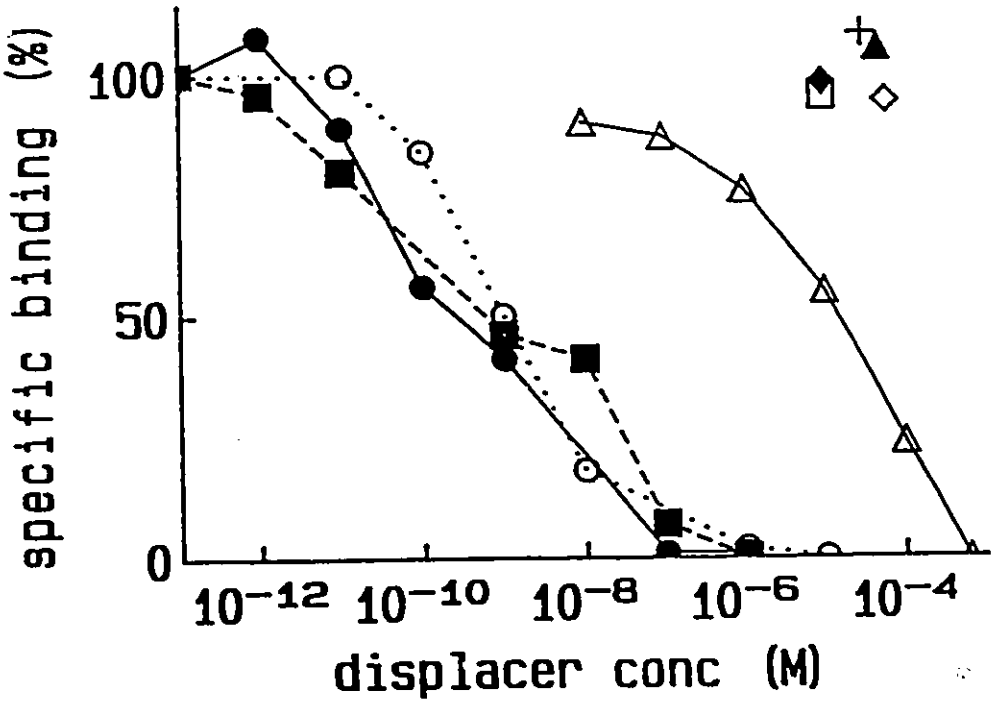

specific binding (%)
100
50
0
10^{-12} 10^{-10} 10^{-8} 10^{-6} 10^{-4}
displacer conc (M)

TABLE 5: Estimates of IC_{50}s and K_is for inhibition of ^{125}I-DAla^{6}EA binding to rabbit pituitary membrane preparations by various agents

Displacer	Log IC_{50} ± SEM	Mean IC_{50}	Mean K_i
DAla^{6}EA-GnRH (7)	-8.87 ± 0.10	1.35 nM	1.12 nM
DLeu^{6}EA-GnRH (3)	-9.17 ± 0.09	0.68 nM	0.56 nM
Gonadorelin Hydrochloride (3)	-7.84 ± 0.23	1.45 nM	1.52 nM
Native GnRH (3)	-5.01 ± 0.21	10.0 μM	8.80 μM
DpGlu^1-GnRH antagonist (4)	-8.85 ± 0.48	1.41 nM	1.74 nM
Vasopressin (2)	--	- -	--
Oxytocin (2)	--	- -	--
PGE_2, $PGF_{2\alpha}$ (2)	--	- -	--
LH-FSH-TSH (2)	--	- -	--

Mean = antilogarithms (geometric means).
IC_{50} values were estimated using the CDATA programme.
$K_i = IC_{50} / \{1 + [L]/K_d\}$
(n) number of experiments

4.4.2.7 *Effect of pH*

Figure 16 shows that a broad pH range of 7 to 8 exists for maximal ^{125}I-DAla^{6}EA binding. Binding was reduced or destroyed at highly acidic or alkaline conditions. A pH of 7.4 at 4°C was used as a standard assay condition.

4.4.2.8 *Tissue Specificity*

To assess tissue specificity of ^{125}I-DAla^{6}EA binding, the 14,000 x g membrane fraction prepared from various rabbit tissues were analyzed for binding (Table 6). ^{125}I-DAla^{6}EA bound to all tissues studied but statistically significant amounts of specific binding were evident in anterior pituitary only ($p<0.05$).

4.4.2.9 *Saturation*

Specific binding of ^{125}I-DAla^{6}EA to rabbit anterior pituitary membrane preparations was found to be saturable at radioligand concentrations of 5 nM and above (Figure 17). The saturation curves were analyzed using a least squares regression analysis for a single and 2 classes of binding sites (BDATA). Estimation of the apparent dissociation constants (K_d) and maximum binding capacities (B_{max}) are shown in Table 7. For 3 out of the 5 experiments data could be satisfactorily fitted by a two site model ($p<0.05$).

FIGURE 16: Effect of pH on specific binding of ^{125}I-DAla^{6}EA. Rabbit pituitary membrane preparations (60-100 μg protein) were incubated with ^{125}I-DAla^{6}EA (0.4 nM) under the assay conditions outlined in Methods. pH was adjusted by the addition of 0.1N HCl or Tris Base to the assay buffer. Data were corrected for NSB determined in the presence of 10^{-6}M unlabelled DAla^{6}EA. Values are means ± SEMs of 3 experiments.

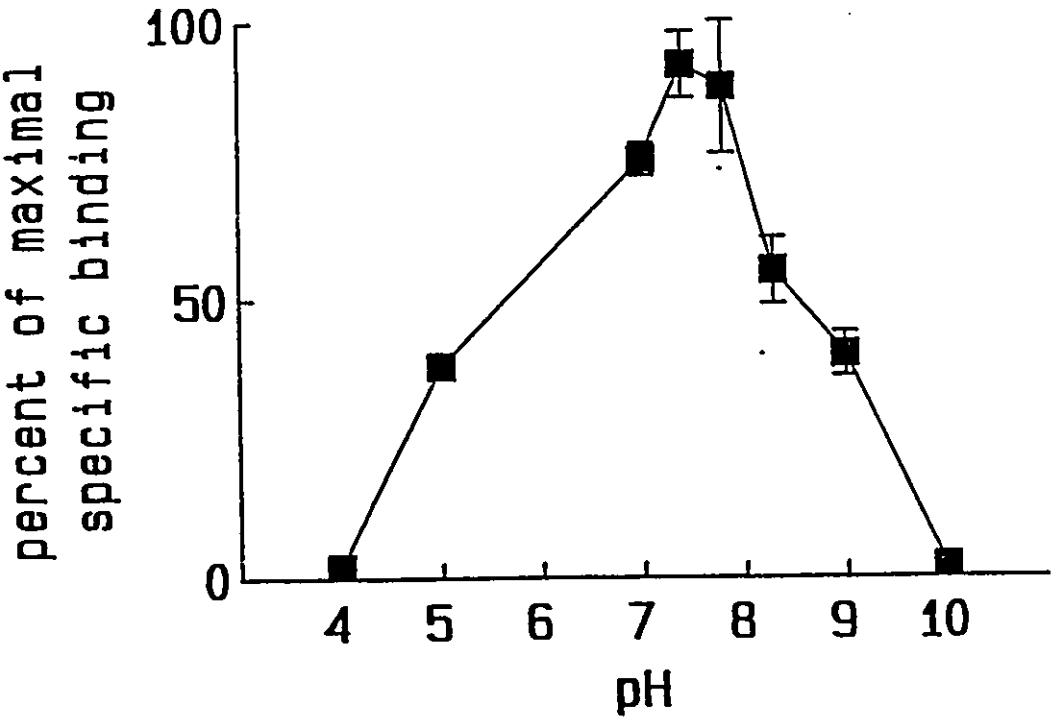

percent of maximal
specific binding
100
50
0
4
5
6
7
8
9
10
pH

TABLE 6: Specificity of ^{125}I-DAla^{6}EA binding to rabbit tissues

Tissue	^{125}I-DAla^{6}EA bound (fmol/mg protein) Total	NSB	Specific
anterior pituitary (11)	137.6± 18.4	43.5± 5.2	94.1
hypothalamus (4)	122.2±21.0	111.8± 17.4	10.4
whole ovary (5)	69.9± 5.4	66.7± 5.4	3.2
liver (2)	149.1± 13.1	186.6±39.8	-
heart (2)	100.2± 1.1	124.9± 19.0	-
spleen (3)	57.2± 5.5	71.8± 12.3	-
kidney (7)	136.9± 16.5	125.4± 12.0	11.5
skeletal muscle (2)	52.6±21.6	51.7±23.5	0.9

Values are means ± SEMs.
NSB was assessed by the addition of 10^{-6}M unlabelled DAla^{6}EA.
Approximately 0.4 nM ^{125}I-DAla^{6}EA added per tube.
70-200 μg protein was used per tube; 14,000 x g membrane preparation.
(n) number of separate experiments; duplicate or triplicate determinations were made at each point.

FIGURE 17: Saturation of ^{125}I-DAla^{6}EA binding of rabbit anterior pituitary membranes. Membrane preparations were incubated with increasing amounts of radiolabelled DAla^{6}EA (0.27 - 9.1 nM) under the assay conditions outlined in Methods. Data show binding corrected for NSB in the presence of 10^{-6}M unlabelled DAla^{6}EA for a representative curve. Specific binding is expressed per fmol/mg protein. Each point is the mean of triplicate determinations.

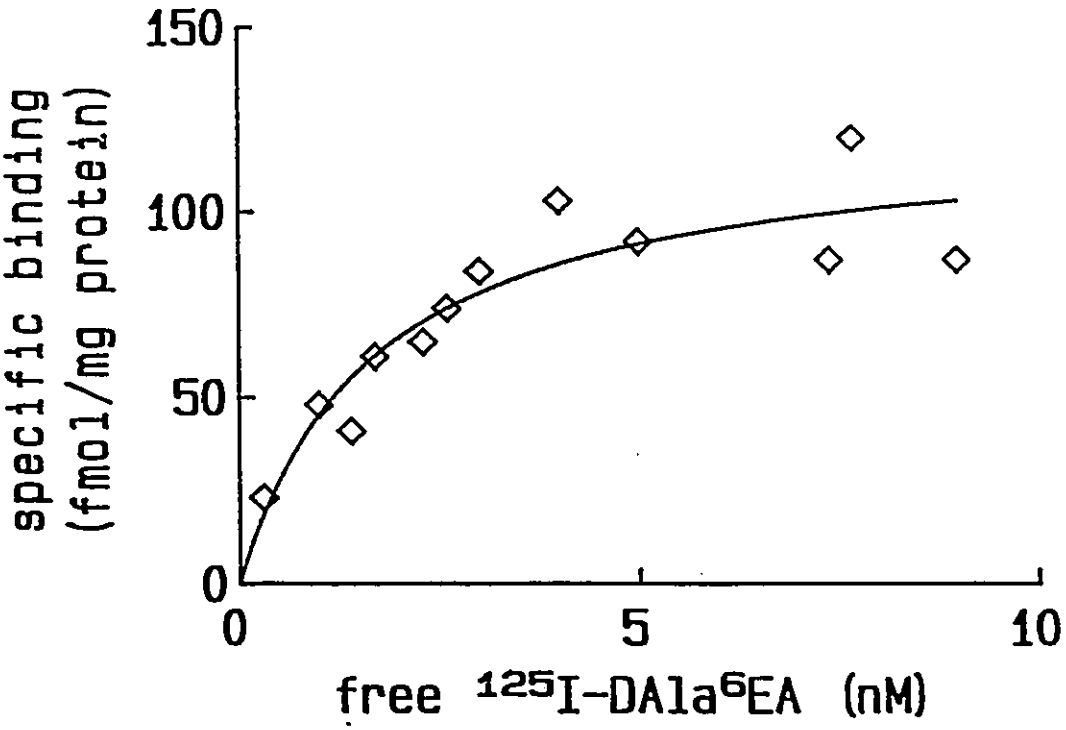

150
100
50
0
specific binding
(fmol/mg protein)
0
5
10
free ^{125}I-DAla^{6}EA (nM)

TABLE 7: Estimates of apparent dissociation constants (K_d) and maximal binding capacity (B_{max}) of ^{125}I-DAla^{6}EA to rabbit anterior pituitary membrane preparations from saturation experiments

	HIGH AFFINITY		LOW AFFINITY	
	K_d (nM)	B_{max} (fmol/mg)	K_d (nM)	B_{max} (fmol/mg)
2 sites*	0.39 ± 0.23	32 ± 10	15.80 ± 3.9	931 ± 244
1 site	3.4 ± 0.95	377 ± 104	- -	- -

Results are the means ± SEMs obtained from analysis of 5 experiments using curves identifying one or two classes of binding sites.
* suitable fit ($p<0.05$) for 3 out of 5 experiments using a 2-site model.

4.4.2.10 *Effect of Extreme Temperature*

Since receptors are glycoproteins they should be destroyed at extreme temperature. Figure 18 shows that when rabbit anterior pituitary membrane preparations were heated at 45-55°C for 10 minutes prior to use in the binding assay, specific binding of ^{125}I-DAla^{6}EA was significantly reduced ($p<0.05$).

4.4.2.11 *Effect of Ions*

The addition of $CaCl_2$ and $MgCl_2$ (0.1 mM, 1.0 mM, 10 mM) to the incubation medium caused a reduction in ^{125}I-DAla^{6}EA binding to pituitary membrane preparations as compared to control tubes ($P<0.05$)(Figure 19A). Similarly, NaCl at concentrations of 30, 150 and 300 mM significantly reduced specific ^{125}I-DAla^{6}EA binding (Figure 19B). The maximum inhibition of the binding was by NaCl.

4.4.2.12 *Effect of Freezing*

Figure 20 shows the effects of freezing rabbit pituitaries (frozen in liquid N_2, stored at -70°C) on specific binding of ^{125}I-DAla^{6}EA. Surprisingly, fresh pituitary preparations had significantly lower binding when compared to frozen membrane preparations ($p<0.05$). No significant change in ^{125}I-DAla^{6}EA binding was evident between 2 weeks, 3 weeks, 4 weeks and 12 weeks of freezing ($p>0.05$). The apparent dissociation constants of fresh (3.38 nM) and frozen (4.44 nM) rabbit pituitaries were similar when data were fitted using a one site binding model.

FIGURE 18: Effect of extreme temperature on ^{125}I-DAla^{6}EA (0.4 nM) specific binding to rabbit anterior pituitaries. Membrane preparations (60-100 μg protein) were heated at 45-55°C for 10 minutes prior to use in the radioligand binding assay. Data were corrected for NSB in the presence of 10^{-6}M unlabelled DAla^{6}EA. Values are means ± SEMs of 3 experiments, 3 observations per experiment (3 total, 3 NSB). Specific binding was significantly reduced when membrane preparations were heated at 45-55°C when compared to 4°C ($p<0.05$).

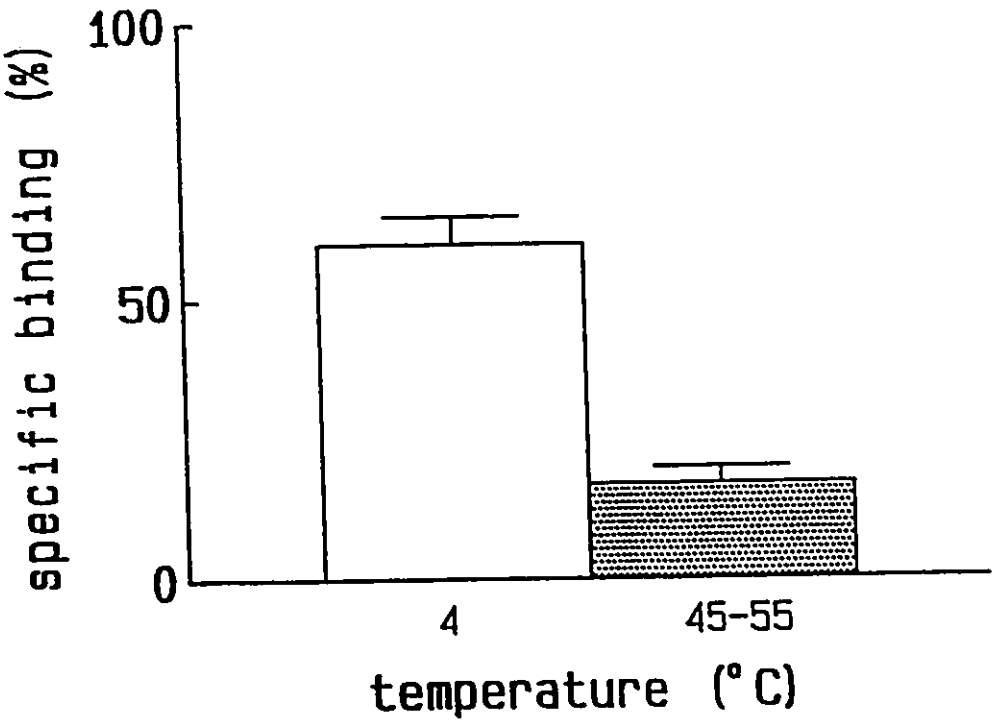

100
50
0
specific binding (%)
4
45-55
temperature (°C)

FIGURE 19A: Effect of calcium and magnesium on ^{125}I-DAla^{6}EA binding to rabbit anterior pituitary membranes. Membrane preparations (60-100 μg protein) were incubated with approximately 0.4 nM labelled DAla^{6}EA with and without 10^{-6}M cold DAla^{6}EA under the incubation conditions described in Methods. Standard assay conditions served as controls. $CaCl_2$ = calcium chloride; $MgCl_2$ = magnesium chloride. Values are means ± SEMs of 3 experiments. A significant decrease in specific binding was evident at all concentrations of $CaCl_2$ and $MgCl_2$ ($p < 0.05$).

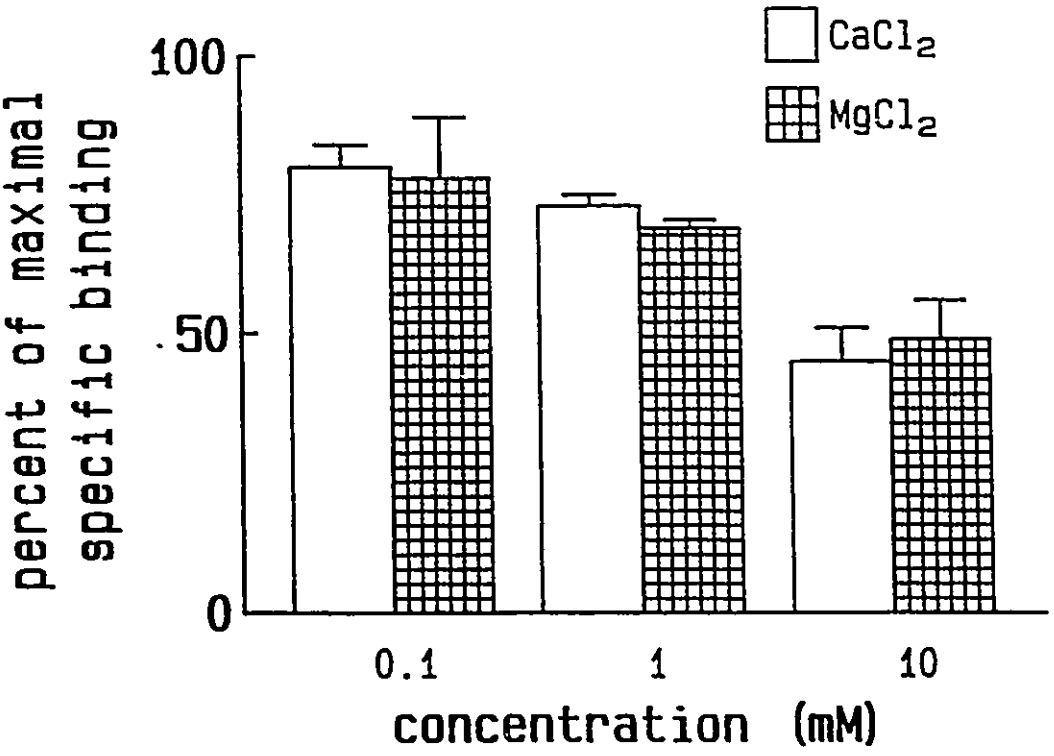

CaCl2
MgCl2
100
50
0
percent of maximal specific binding
0.1
1
10
concentration (mM)

FIGURE 19B: Effects of sodium and sucrose on specific binding of ^{125}I-DAla^{6}EA to rabbit anterior pituitary membranes. Membrane preparations were incubated with 0.4-0.6 nM labelled DAla^{6}EA with and without 10^{-6}M unlabelled DAla^{6}EA under the incubation conditions described in Methods. NaCl and sucrose were added to the assay buffer for final concentrations of 30, 150 and 300 mM. Standard assay conditions served as controls. The effect of sucrose on binding was included to identify changes due to alterations in osmotic concentration. Values are the means ± SEMs of 3 (sodium) or 4 (sucrose) experiments, each carried out in triplicate. NaCl = sodium chloride. A significant decrease ($p<0.05$) in specific binding was evident at all concentrations of NaCl and at concentrations 150 mM and 300 mM of sucrose.

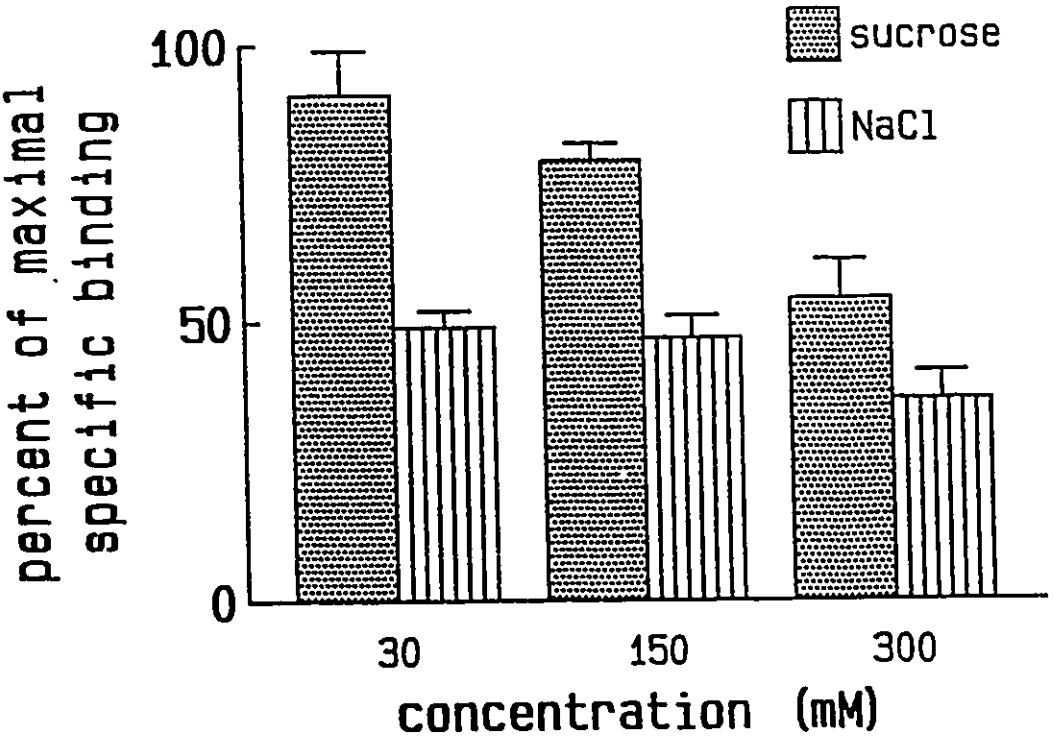
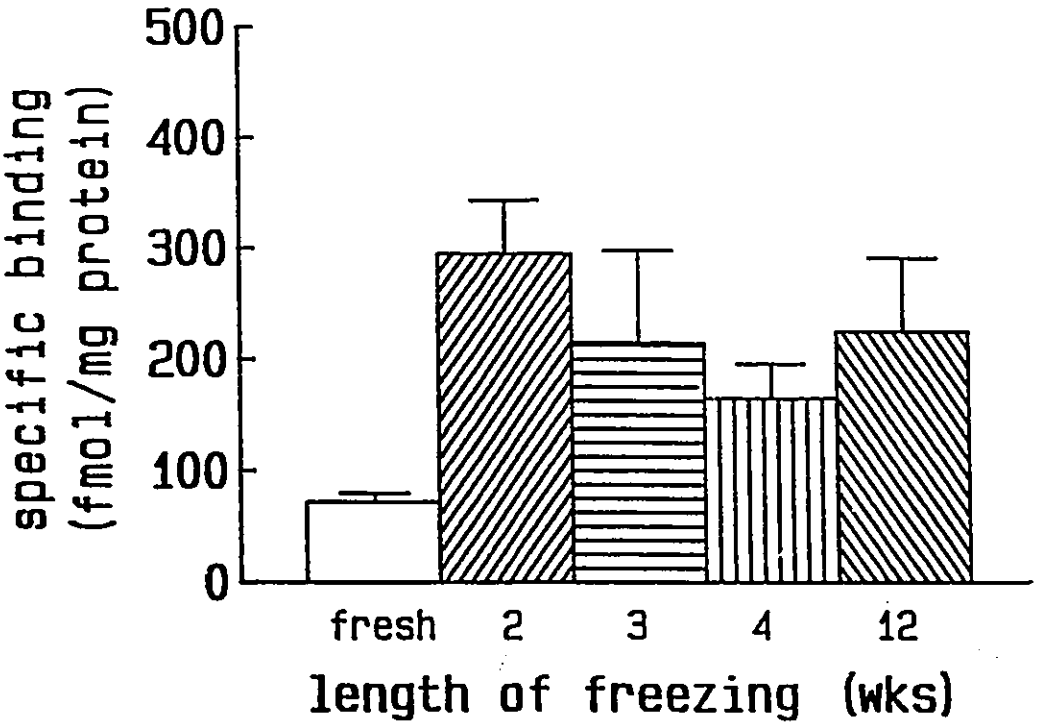

sucrose
NaCl
100
50
0
percent of maximal specific binding
30
150
300
concentration (mM)

FIGURE 20: Effect of freezing (quick frozen in liquid nitrogen, stored at -70°C) on specific binding of ^{125}I-DAla^{6}EA. Rabbit pituitary membrane preparations were incubated with ^{125}I-DAla^{6}EA (approximately 0.4 nM) according to the assay conditions outlined in Methods. Values are means ± SEMs. Each determination was carried out in triplicate (3 total, 3 NSB). NSB was assessed in the presence of 10^{-6}M unlabelled DAla^{6}EA. Number of determinations in parentheses. ^{125}I-DAla^{6}EA binding to fresh preparations was significantly lower than frozen preparations ($p<0.05$). Specific binding of frozen tissues were not significantly different from one another ($p>0.05$).


specific binding
(fmol/mg protein)
500
400
300
200
100
0
fresh
2
3
4
12
length of freezing (wks)

4.4.2.13 *Tracer Degradation*

The extent of degradation of labelled DAla^{6}EA after initial incubation with anterior pituitary membrane preparations is shown in Table 8. The ability of the unbound tracer (^{125}I-DAla^{6}EA) in the supernatant following incubation with pituitary membrane preparations to bind to a fresh aliquot of membrane preparation was measured. Control tubes measured binding to a fresh aliquot of membrane preparation of the radioligand previously incubated without membrane. Degradation of the radioligand was not significant at 60 or 120 minutes ($p > 0.05$).

4.4.2.14 *Kinetics*

The specific binding of ^{125}I-DAla^{6}EA to pituitary membrane preparations increased with time and reached equilibrium by 45 minutes (Figure 21A). At E_q the concentration of label bound was 2-3 percent of the total DAla^{6}EA label added. Thus, free ligand and total ligand can be considered equal. The plot of ln ($B_{Eq}/B_{Eq} - B_t$) versus time gave a straight line as shown in Figure 21B. Addition of an excess (10^{-6}M) of unlabelled DAla^{6}EA resulted in a rapid dissociation of specific binding (see Figure 21A). Dissociation occurred quickly for approximately 10 minutes followed by a slower component (Figure 21C). This biphasic dissociation may be due to the presence of two binding sites.

The association curve led to an observed association rate (k_{obs}) of .072 per minute. The first order dissocation constant (k_2) was 0.18 per minute. The association

TABLE 8: Degradation of radiolabelled DAla^{6}EA peptide by pituitary membrane preparations incubated at 4°C. The extent of degradation was determined by the ability of the unbound tracer in the supernatant (following 60 or 120 minutes of incubation) to bind to a fresh aliquot of pituitary membrane preparations

Incubation Time	Percent of Control Specific Binding
60 minutes [a]	101.7 ± 8.0
120 minutes [b]	100.5 ± 27.8

Results shown are the means ± SEMs of 3 determinations, each carried out in triplicate.
Control = initial incubation without membrane preparation.
Approximately 0.4 nM ^{125}I-DAla^{6}EA was added per tube.
[a] Binding of unbound tracer from initial 60 min incubation to a fresh aliquot of membrane preparation.
[b] Binding of unbound tracer from initial 120 min incubation to a fresh aliquot of membrane preparation.
Specific binding was not significantly different from control specific binding ($p > 0.05$).

FIGURE 21A: Reversibility of ^{125}I-DAla^{6}EA binding to rabbit anterior pituitary membrane preparations (60-100 μg protein). Following 60 minutes of incubation (approximately 0.4 nM ^{125}I-DAla^{6}EA) an excess (10^{-6}M) of unlabelled DAla^{6}EA was added as indicated by the arrow. Binding was terminated at the indicated time periods by centrifugation (11,000 x g, 15 min). Values are means ± SEMs of 3 experiments. ● = total binding; o = NSB.

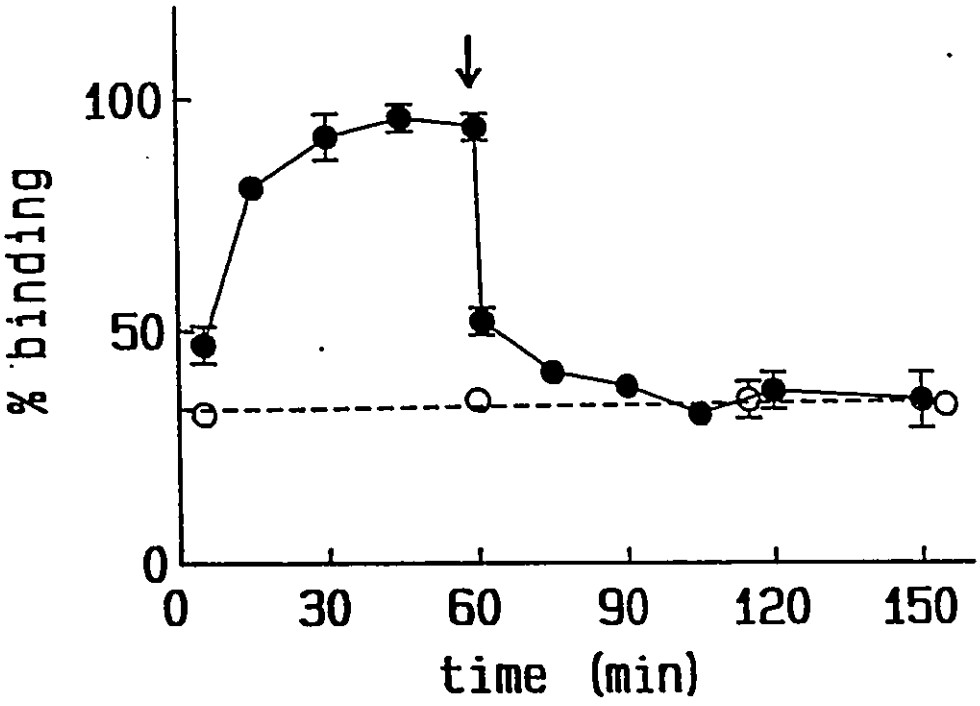

100
50
0
% binding
0
30
60
90
120
150
time (min)

FIGURE 21B: Association rate of ^{125}I-DAla^{6}EA binding to rabbit anterior pituitary membrane preparations. Incubations were carried out at various time intervals up to 60 minutes and terminated by filtration. The data are plotted as ln $[B_{Eq}/B_{Eq}-B_t]$ versus time with a slope of k_{obs} (.072 min^{-1}). The association rate constant (k_1) was 1.35 x 10^8M per minute calculated from the equation $k_1 = (k_{obs} - k_2)[L]^{-1}$ where [L] = 0.4 nM ^{125}I-DAla^{6}EA.

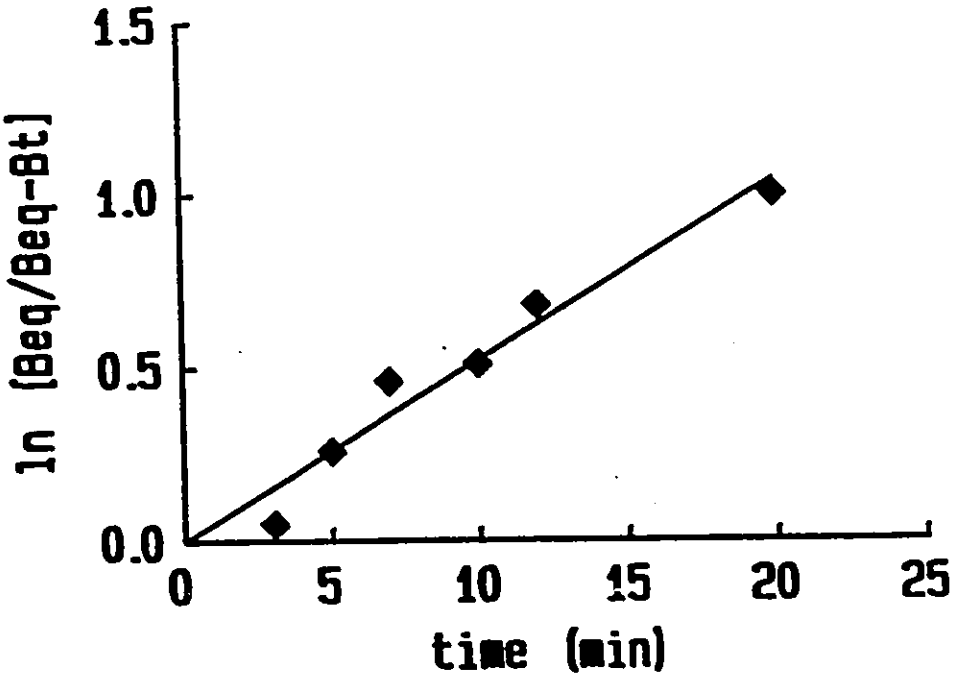
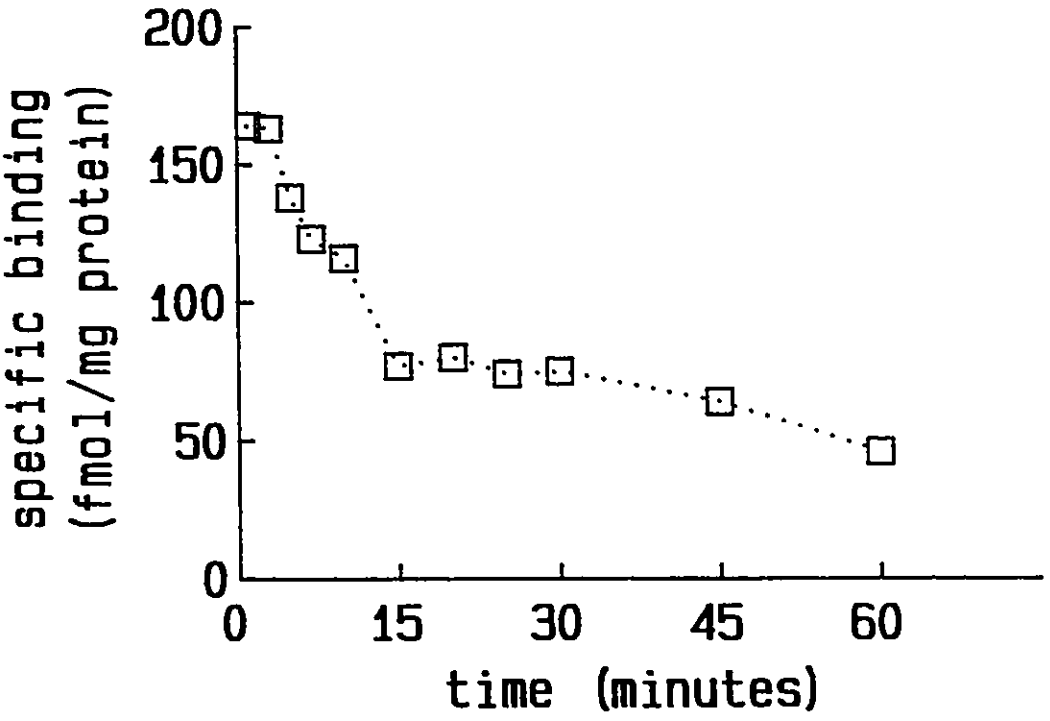

1.5
1.0
0.5
0.0
ln [Beq/Beq-Bt]
0
5
10
15
20
25
time (min)

FIGURE 21C: Dissociation rate of ^{125}I-DAla^{6}EA binding to rabbit anterior pituitary membrane preparations. Following 60 minutes of incubation with approximately 0.4 nM ^{125}I-DAla^{6}EA, dissociation was initiated by the addition of an excess (10^{-6}M) of unlabelled DAla^{6}EA. Incubations were terminated at various time intervals up to 60 minutes by filtration. The data are plotted as specific binding (fmol/mg protein) determined at the various time intervals. The dissociation rate constant (k_2) was 0.18 min^{-1} (estimated from the slope of the regression line multiplied by -2.303).


specific binding
(fmol/mg protein)
200
150
100
50
0
0
15
30
45
60
time (minutes)

rate constant (k_1) calculated from the equation $k_1 = k_{obs}\text{-}k_2[^{125}\text{I-DAla}^6\text{EA}]^{-1}$ was 1.35 x 10^8 M per minute. A kinetically derived dissociation constant K_d calculated from these values (k_2/k_1) was 0.14 nM.

4.4.2.15 *Displacement*

Addition of 10^{-6} M unlabelled DAla^{6}EA resulted in displacement of 55-67 percent of the totally bound ^{125}I-DAla^{6}EA. For each assay, non-displaceable binding in the presence of 10^{-6}M unlabelled DAla^{6}EA was determined and used for subtraction of non-specific binding.

Detailed competition curves for DAla^{6}EA and DpGlu^1 were conducted over the extended range of concentrations 10^{-15} M to 10^{-5} M. Curves were fitted separately using the nonlinear computerized CDATA programme for a one class or two classes of binding sites. DAla^{6}EA could be fitted ($p<0.05$) to a curve describing two classes of binding sites in 2 of the 4 experiments. DpGlu^1 displacement was suitably fitted ($p<0.05$) using a 2 site model in 3 out of the 5 experiments (Table 9). Analysis of the pooled data from these same experiments resulted in a significant fit using the two site model for DpGlu^1 ($p<0.05$) but not for DAla^{6}EA ($p>0.05$).

TABLE 9: Estimates of equilibrium binding constants for DAla^{6}EA and DpGlu^1 from displacement data

	ONE SITE (nM)	TWO SITES (nM)	(nM)
DAla^{6}EA [a]	1.37 ± 0.18	854 ± 71	0.85 ± 0.10
DpGlu^1 [b]	2.04 ± 0.85	8.6 ± 4.20	0.02 ± 0.01

Results are the means ± SEMs from analysis of 1 or 2 classes of binding sites using the CDATA programme.
Approximately 0.4 nM ^{125}I-DAla^{6}EA added per tube.
[a] suitable fit ($p<0.05$) for 2 out of 4 experiments using a two site model.
[b] suitable fit ($p<0.05$) for 3 out of 5 experiments using a two site model.

4.4.3 DOWN REGULATION OF ^{125}I-DALA^{6}EA BINDING

The effects of in vivo injection of 100 ng/kg of a GnRH antagonist (DpGlu^1) or agonist (DAla^{6}EA) every 12 hours for 4 days is shown in Figure 22. Injection of the GnRH agonist DAla^{6}EA resulted in a 80 percent decrease in ^{125}I-DAla^{6}EA binding when compared with controls ($p<0.05$) using single point binding analysis. Although DpGlu^1 injection caused a slight increase in ^{125}I-DAla^{6}EA binding when compared with controls, this was not significant ($p>0.05$). Body weights, pituitary weights and serum LH were not significantly different between groups ($p>0.05$) (Table 10). Saturation analysis fitted for one site demonstrated similar apparent dissociation constants (K_d) between treatment groups (control: K_d=3.28 nM; DAla^{6}EA: K_d=4.27 nM; DpGlu^1: K_d=3.05 nM)(see Table 10).

4.4.4 ONTOGENY OF ^{125}I-DALA^{6}EA BINDING

The binding of ^{125}I-DAla^{6}EA to anterior pituitaries (by single point analysis) of female rabbits of varying ages is depicted in Figure 23. ^{125}I-DAla^{6}EA binding increased from day 25 (44.78 ± 10.09 fmol/mg protein) to a maximal value at day 85 (268.8 ± 71.06 fmol/mg protein). At 120 days of age, the amount of binding was reduced (132.5 ± 10.72 fmol/mg protein) when compared to 85 days of age but this was not significant ($p>0.05$). A significant increase in ^{125}I-DAla^{6}EA binding was obtained between 40 and 50 days of age ($p<0.05$).

Body weights, anterior pituitary weights and serum LH values from each age

FIGURE 22: Down regulation of specific binding of ^{125}I-DAla^{6}EA. Adult rabbits were injected with 100 ng/kg of each drug every 12 hours (8am:8pm) for 4 days. Control = saline; DpGlu^{1}EA = GnRH antagonist; DAla^{6}EA = GnRH agonist; 12hr = 12 hours following a single injection of DAla^{6}EA. Specific binding is expressed per fmol/mg protein. Single point binding analysis (approximately 0.8 nM ^{125}I-DAla^{6}EA) was conducted on individual pituitaries. Number of animals in parentheses. Specific binding was significantly reduced following DAla^{6}EA injection when compared to control rabbits ($p<0.05$).

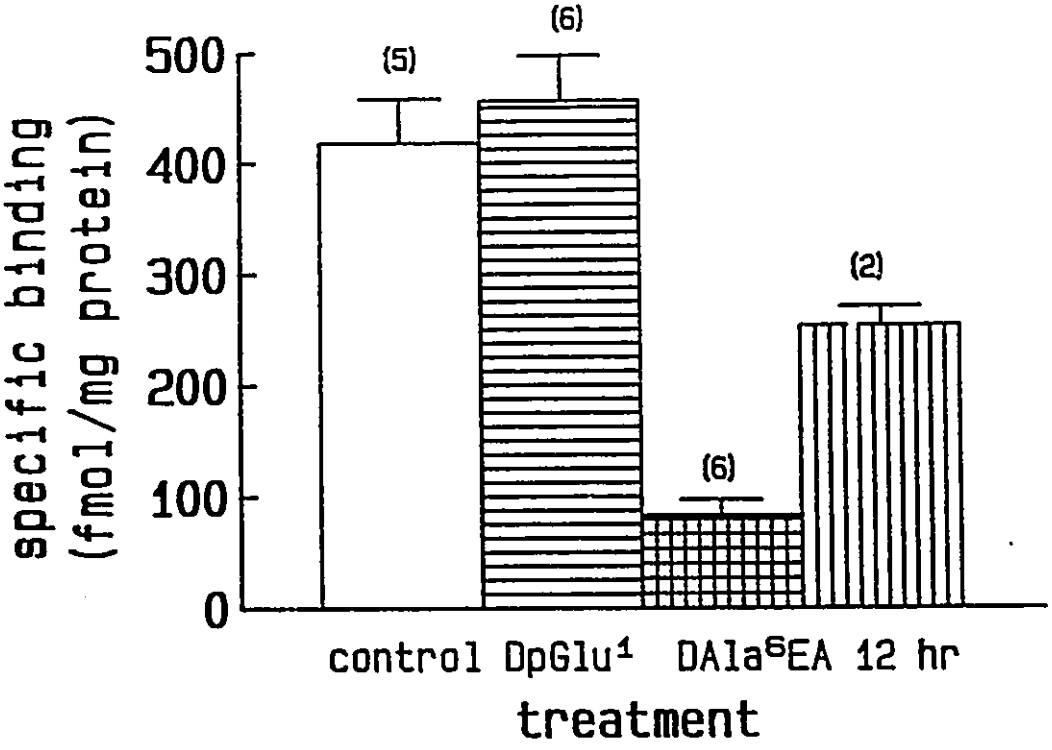

specific binding
(fmol/mg protein)
500
400
300
200
100
0
(5)
(6)
(6)
(2)
control
DpGlu¹
DAla⁶EA
12 hr
treatment

TABLE 10: Serum LH, pituitary weights, body weights and pituitary ^{125}I-DAla^{6}EA binding of adult female rabbits following subcutaneous injection of GnRH analog (DAla^{6}EA) or GnRH antagonist (DpGlu^1) every 8 hours for 4 days

Condition	LH (ng/ml)	Body Weight (g)	Pituitary Weight (mg)	K_d (nM)	B_{max} (fmol/mg protein)
control (6)	0.44 ± 0.02	4590 ± 86	50.17 ± 6.16	3.28	1260
DpGlu^1 (7)	0.45 ± 0.03	4270 ± 279	37.71 ± 3.91	3.05	970
DAla^{6}EA (7)	0.56 ± 0.07	4185 ± 229	41.57 ± 2.14	4.27	526

(n) number of rabbits per group.
Values are means ± SEMs.
K_d and B_{max} were determined from saturation analysis, fitted using a one site model, one determination only.
LH, body weights and pituitary weights were not significantly different across treatment groups ($p<0.05$).

group are shown in Table 11. Body weight and pituitary weight increased throughout maturation. Body weight increases in all age groups were significantly different from each other ($p<0.05$) except between 75 and 85 days of age ($p>0.05$). A significant increase in pituitary weight was evident between 40 and 50 days as well as 85 and 120 days of ages. The serum LH pattern showed a peak at 40-50 days of age followed by a decline by 75 days of age.

4.4.5 ^{125}I-DALA^{6}EA BINDING CONSTANTS TO FEMALE RABBIT PITUITARIES (40, 75 AND 120 DAYS OF AGE)

^{125}I-DAla^{6}EA binding as a function of increasing concentrations of the radioligand for pituitary membranes of rabbits aged 40, 75 and 120 days is illustrated in Figures 24A, 25A and 26A. Results from analysis using the BDATA progamme fitting for one binding site is shown in Table 12A. The data were better fitted by a two site model as compared to a one site model in several experiments (Table 12B). The high affinity sites in all age groups were similar (K_d = 0.3 to 0.4 nM). The low affinity sites for 75 and 120 days were similar at a K_d of approximately 30 nM while those at 40 days were of much lower affinity (150 nM).

FIGURE 23: Ontogeny of ^{125}I-DAla^{6}EA binding to female rabbit anterior pituitary membrane preparations. Data were corrected for NSB in the presence of 10^{-6}M unlabelled DAla^{6}EA. Values are means ± SEMs of 3 animals per group. Single point binding analysis (approximately 0.4-0.6 nM labelled DAla^{6}EA) was conducted on individual pituitaries of each age group.

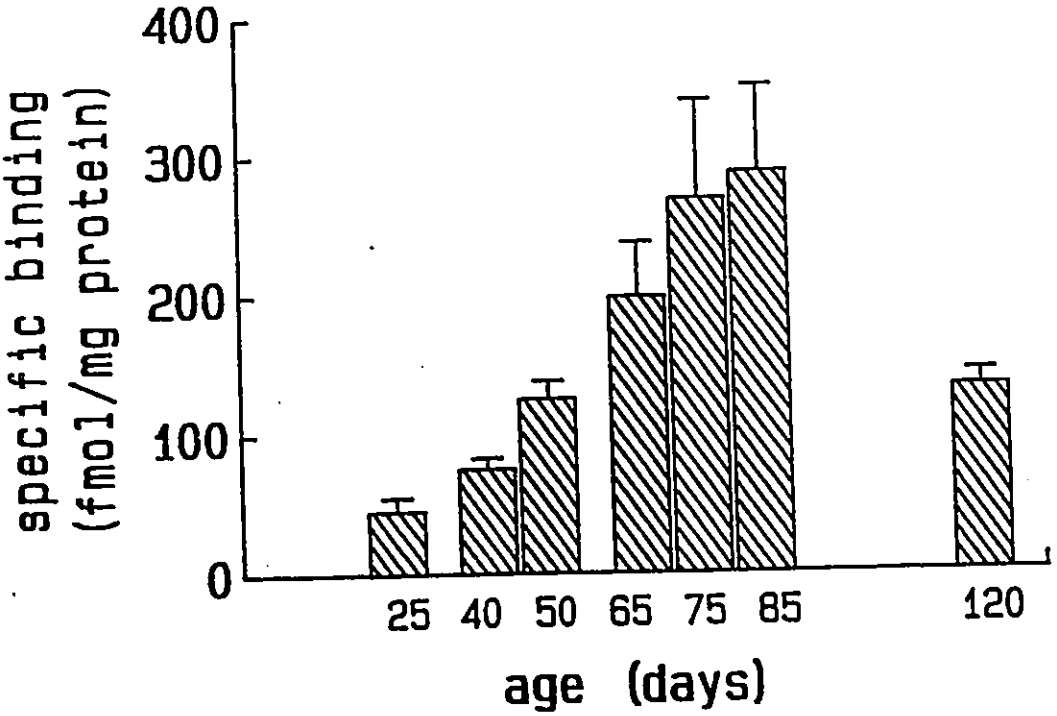

specific binding
(fmol/mg protein)
400
300
200
100
0
25
40
50
65
75
85
120
age (days)

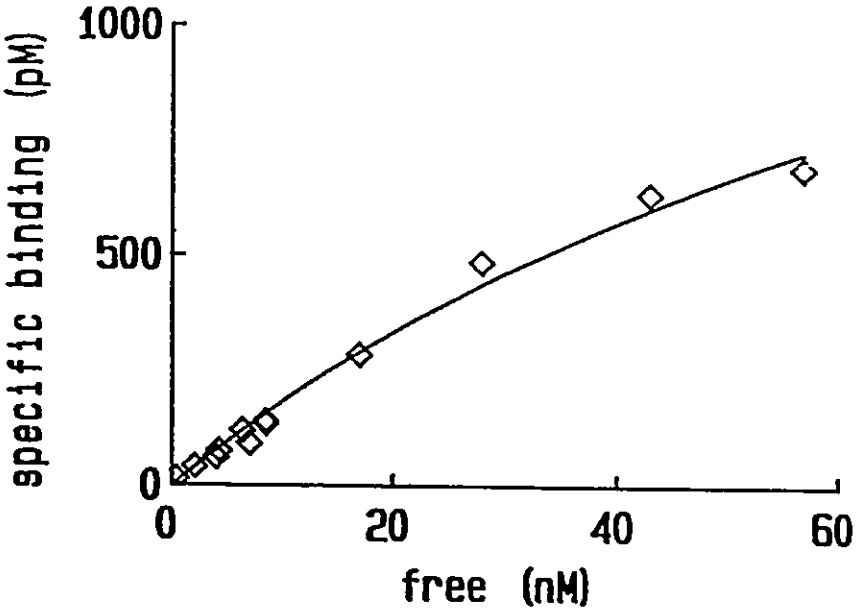
TABLE 11: Age-related changes in serum luteinizing hormone (LH), body weight and anterior pituitary weight of female rabbits

Age (days)	LH (ng/ml)	Body Weight (g)	Pituitary Weight (mg)
25	0.43 ± 0.01	475 ± 21	12 ± 2
40	1.27 ± 0.33*	1168 ± 71*	17 ± 2
50	2.28 ± 1.04*	1613 ± 56*	28 ± 2*
65	NR	2148 ± 70*	25 ± 1
75	0.51 ± 0.07*	2563 ± 135*	33 ± 4
85	0.53 ± 0.09	2911 ± 79	31 ± 4
120	0.58 ± 0.10	3808 ± 258*	49 ± 1*

Values are the means ± SEMs for 3 rabbits.
NR = not reported
* $p<0.05$ when compared to previous age group.

FIGURE 24A: Representative curve of ^{125}I-DAla^{6}EA binding to 40 day female rabbit anterior pituitary membrane preparations as a function of increasing concentrations of the ligand (0.2 - 60 nM ^{125}I-DAla^{6}EA). Data show binding corrected for non specific binding. NSB was assessed in the presence of 10^{-6}M unlabelled DAla^{6}EA. Results are from two experiments, each carried out in triplicate (3 total, 3 NSB).

FIGURE 24B: Scatchard plot of ^{125}I-DAla^{6}EA binding to 40 day rabbit anterior pituitary membrane preparation. Plot was derived from the data of Figure 24A.


specific binding (pM)
1000
500
0
0
20
40
60
free (nM)

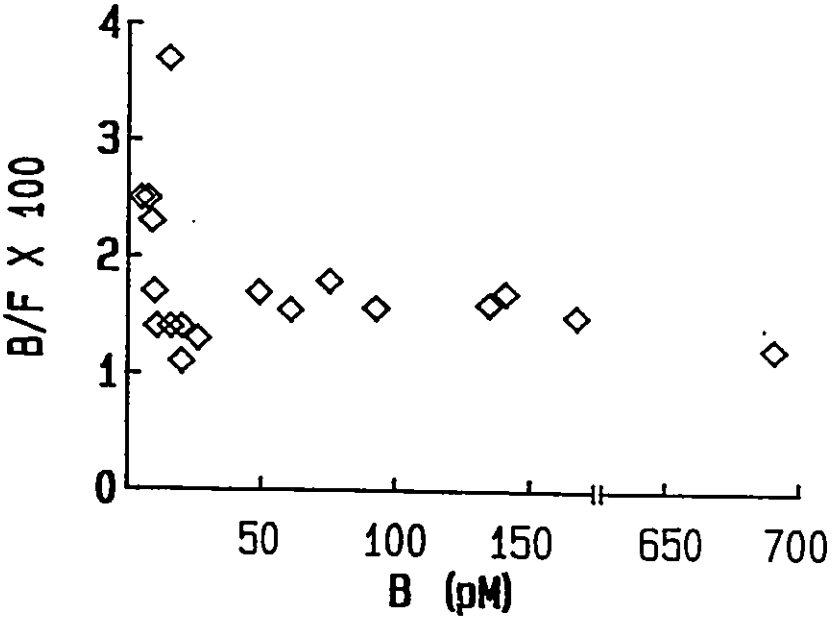
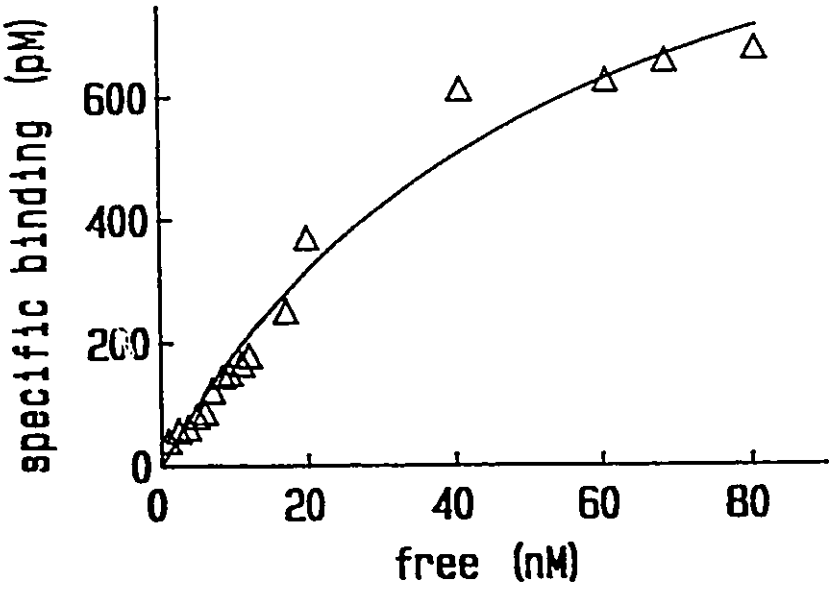

B/F X 100
4
3
2
1
0
50
100
150
650
700
B (pM)

FIGURE 25A: Representative curve of ^{125}I-DAla^{6}EA binding to 75 day female rabbit anterior pituitary membrane preparations as a function of increasing concentrations of the ligand (0.2 - 80 nM ^{125}I-DAla^{6}EA). Data show binding corrected for non specific binding. NSB was assessed in the presence of 10^{-6}M unlabelled DAla^{6}EA. Results are from two experiments, each carried out in triplicate (3 total, 3 NSB).

FIGURE 25B: Scatchard plot of ^{125}I-DAla^{6}EA binding to 75 day rabbit anterior pituitary membrane preparation. Plot was derived from the data of Figure 25A.


specific binding (pM)
600
400
200
0
0
20
40
60
80
free (nM)

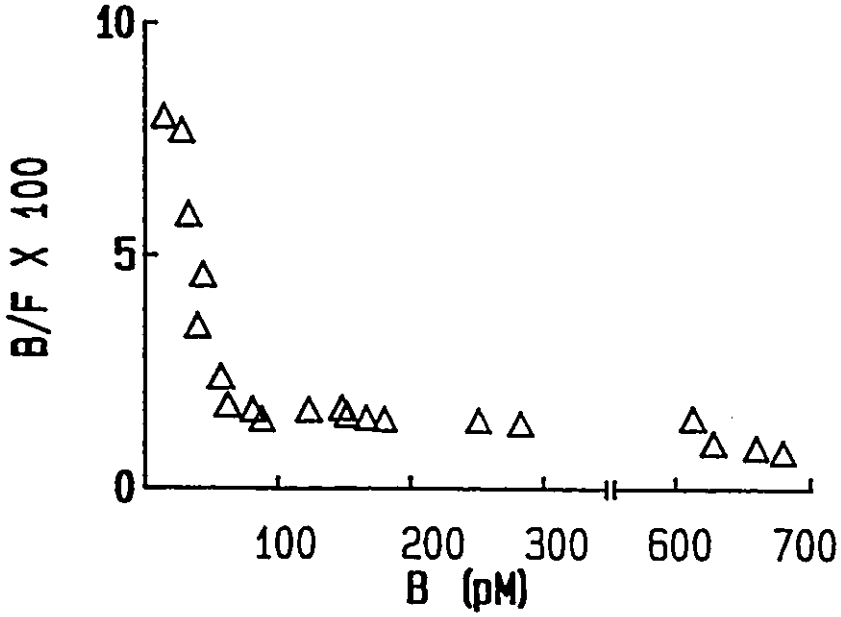
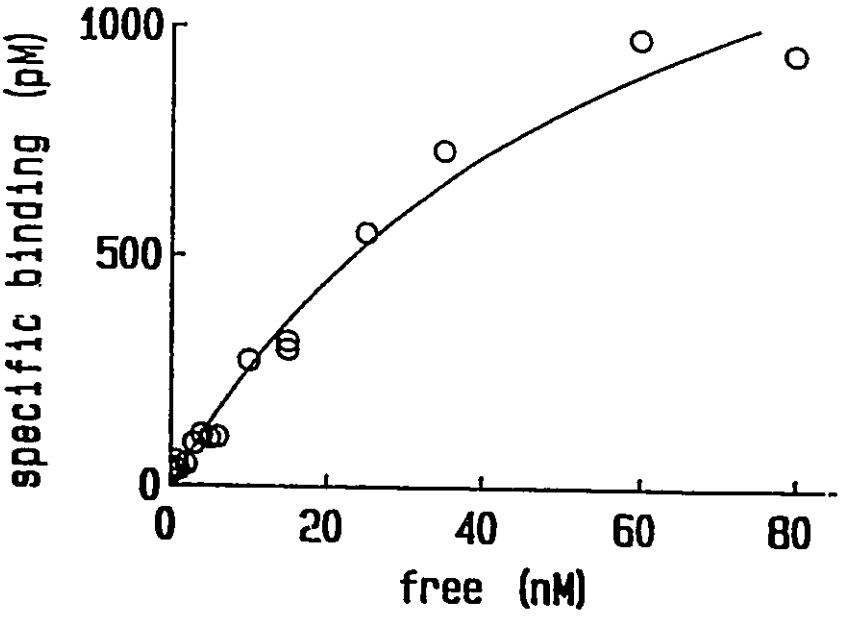

B/F X 100
10
5
0
100
200
300
600
700
B (pM)

FIGURE 26A: Representative curve of ^{125}I-DAla^{6}EA binding to 120 day female rabbit anterior pituitary membrane preparations as a function of increasing concentrations of the ligand (0.2 - 80 nM ^{125}I-DAla^{6}EA). Data show binding corrected for non specific binding. NSB was assessed in the presence of 10^{-6}M unlabelled DAla^{6}EA. Results are from two experiments, each carried out in triplicate (3 total, 3 NSB).

FIGURE 26B: Scatchard plot of ^{125}I-DAla^{6}EA binding to 120 day rabbit anterior pituitary membrane preparation. Plot was derived from the data of Figure 26A.


specific binding (pM)
1000
500
0
0
20
40
60
80
free (nM)

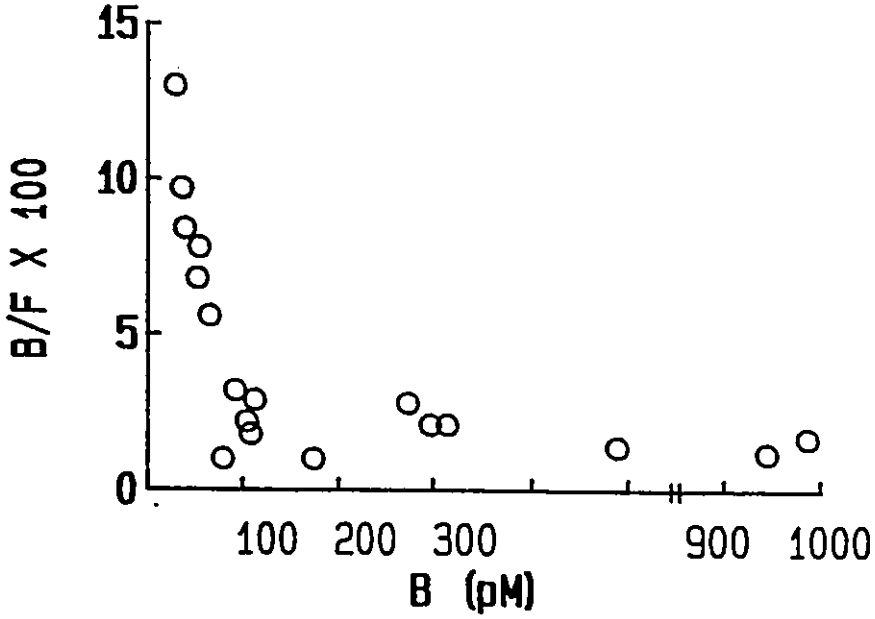

B/F X 100
15
10
5
0
100
200
300
900
1000
B (pM)

TABLE 12A: Estimates of apparent dissociation constants (K_d) and maximal binding capacities (B_{max}) of ^{125}I-DAla^{6}EA to female rabbit anterior pituitary membrane preparations using a one site model

Age (days)	K_d (nM)	B_{max} (fmol/mg protein)
40 (3)	15.93 ± 5.89	2312 ± 475
75 (3)	7.76 ± 1.78	3443 ± 1820
120 (5)	5.83 ± 0.69	2666 ± 511

Data were derived from saturation curves.
Estimates were derived from curves fitted for one site according to the BDATA programme.
(n) number of experiments.
Results are the means ± SEMs.

TABLE 12B: Estimates of apparent dissociation constants (K_d) and maximal binding capacities (B_{max}) of ^{125}I-DAla^{6}EA to female rabbit anterior pituitary membrane using a two-site model

Age (days)	High Affinity		Low Affinity	
	K_d (nM)	B_{max} (fmol/mg)	K_d (nM)	B_{max} (fmol/mg)
40[a] (3)	0.35± 0.09	27±10	155.3±62.4	14711±4102
75[b] (3)	0.32± 0.17	107±69	31.7±10.0	8208±4532
120[c] (5)	0.44± 0.28	164±37	28.2± 9.2	5567±1739

Data were derived from saturation curves.
Values were estimated using the BDATA programme, curves fitted for 2 binding sites.
(n) number of experiments
Values are the means ± SEMs.
[a] suitable fit ($p<0.05$) for 1 out of 3 experiments.
[b] suitable fit ($p<0.05$) for 2 out of 3 experiments.
[c] suitable fit ($p<0.05$) for 3 out of 5 experiments.

4.5 IN VIVO LH RESPONSE TO DALA^{6}EA

The LH response over time to 3 concentrations of DAla^{6}EA (10 ng, 100 ng and 1 μg per kg body weight) for female rabbits aged 40, 75 and 120 days of age is shown in Table 13. The administration of DAla^{6}EA led to a prompt increase in LH for the three age groups studied at all dosages. Generally, the increase was most dramatic by 30 minutes and by 45 minutes began to decline in rabbits 40 and 75 days of age. At 120 days, the increase in LH was more sustained throughout the 120 minutes.

After administration of 10 ng/kg DAla^{6}EA, the increase in LH was significant when compared to baseline LH values in 40 day and 120 day rabbits ($p<0.05$)(see Table 13). In addition, the increase in LH remained significant in the 120 day rabbits at 120 minutes following DAla^{6}EA administration at 10 ng/kg.

Administration of 100 ng/kg DAla^{6}EA resulted in significant increments in LH (Δ LH at 30 minutes, Table 14) for female rabbits aged 40 days (13.3 $\pm$ 1.99 ng/ml), 75 days (18.55 $\pm$ 3.4 ng/ml) and 120 days (18.7 $\pm$ 3.59 ng/ml) ($p<0.05$). Similarly, injection of 1 μg/kg DAla^{6}EA produced significant increases in LH at 30 minutes in all age groups (40 day = 18.23 $\pm$ 4.85 ng/ml; 75 day = 34.66 $\pm$ 8.21 ng/ml; 120 day = 26.23 $\pm$ 6.76 ng/ml). Due to the high variability of the data, there were no significant differences in LH responsiveness 30 minutes post injection to 100 ng and 1 μg/kg DAla^{6}EA between 40 day, 75 day and 120 day female rabbits ($p>0.05$).

TABLE 13: Changes in peripheral LH concentrations (ng/ml) in female rabbits (40, 75 and 120 days of age) after a single injection of DAla^{6}EA

Dosage	Time (minutes) 0	15	30	45	120
10ng/kg					
40	1.16±0.27	1.74±0.37	3.91±1.06*	3.39±0.82*	1.54±0.39
75	0.42±0.00	0.44±0.02†	0.74±0.17	0.79±0.15	0.49±0.04
120	0.76±0.15	1.40±0.22	3.14±1.33*	4.90±2.03*	12.17±5.12*†
100ng/kg					
40	0.57±0.05	7.70±1.74*	14.53±1.98*	17.46±2.89*	3.08±0.86
75	0.55±0.11	7.93±3.44*	19.10±3.49*	18.08±1.95*	9.19±3.36*
120	0.50±0.07	10.63±4.60*	19.21±3.57*	23.62±2.41*	25.46±2.68*†
1μg/kg					
40	0.50±0.07	17.12± 5.18*	19.42±4.84*	14.90±6.18*	4.18±0.98
75	0.56±0.08	23.84± 6.72*	35.16±8.23*	29.79±6.92*	12.59±3.12*
120	0.47±0.04	12.60± 2.53*	26.70±6.76*	29.27±7.18*	27.26±6.42*†

Values are means ± SEMs for 5-7 rabbits.
* $p<0.05$ compared with sample at time 0 (basal level).
† $p<0.05$ compared within dosage, between all ages at each sampling time (Scheffés comparison).

TABLE 14: Body weights and mean differences in luteinizing hormone (LH) before and 30 minutes (peak values) after DAla^{6}EA administration to female rabbits

Dosage	Age (days)	Weight (g)	Δ LH (ng/ml)
10ng/kg	40	1289 ± 42	2.81 ± 1.08‡‡
	75	2163 ± 90†	0.32 ± 0.02‡‡
	120	3397 ± 163†	2.38 ± 1.40‡‡
100ng/kg	40	1355 ± 62	13.33 ± 1.99*
	75	2317 ± 49†	18.55 ± 3.43*
	120	3270 ± 198†	18.70 ± 3.59*
1μg/kg	40	1488 ± 50	18.23 ± 4.85*
	75	2369 ± 84†	34.66 ± 8.21*
	120	3920 ± 150†	26.23 ± 6.76*

Values are means ± SEMs of 5-7 animals.
* Significant increases in LH when compared to baseline LH values ($p<0.05$).
† $p<0.05$ compared with previous age group.
‡‡ $p<0.05$ compared within each age group between all dosages (Scheffés comparison).

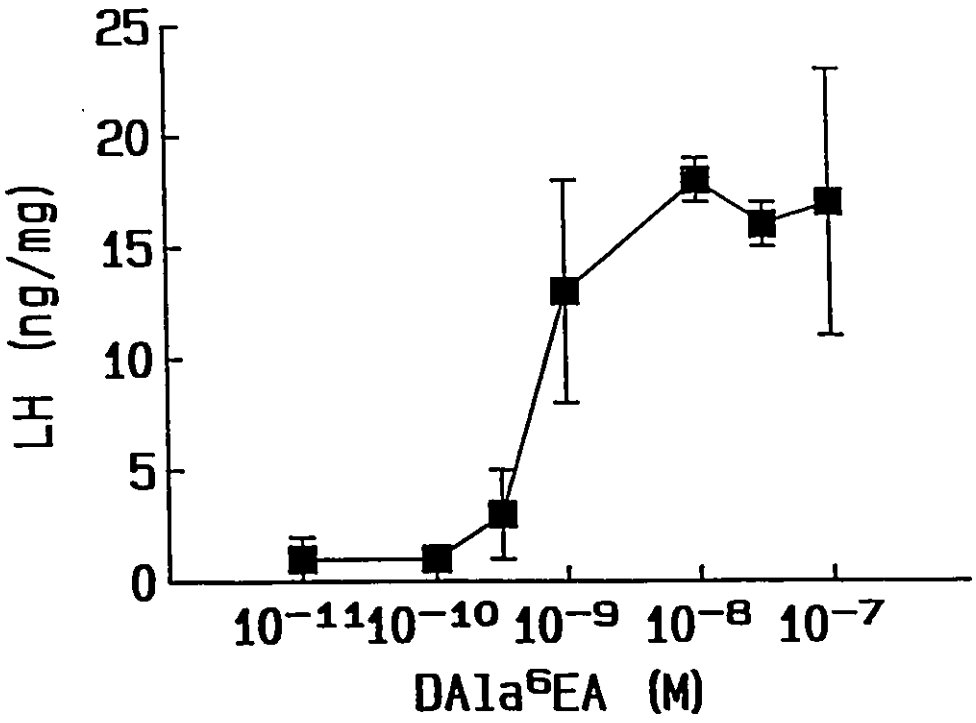
The difference in dose-effect between 10 ng/kg and 100 ng/kg or 1 μg/kg of DAla^{6}EA was significant ($p<0.05$) in all three age groups studied (Table 14).

Table 14 shows the body weights of animals 40, 75 and 120 days of age. Body weights were significantly different across each age group ($p<0.05$). Body weights within each group were similar across all 3 dosages of DAla^{6}EA ($p>0.05$).

4.6 IN VITRO LH RESPONSE TO GnRH AND GnRH ANALOGS

Figure 27 shows the in vitro LH response to DAla^{6}EA over the concentration 10^{-11}M to 10^{-7}M. Exposure of female rabbit pituitary slices to increasing concentations of DAla^{6}EA resulted in a dose-dependent stimulation of LH. Using six dosages of DAla^{6}EA the ED_{50} was estimated as 0.6 x 10^{-9}M. The rank order of potency for GnRH and a number of GnRH analogs was DAla^{6}EA = DLeu^{6}EA > Factrel > native GnRH. However, due to the high variability of the data, accurate estimations of the ED_{50}s for GnRH analogs could not be carried out.

FIGURE 27: In vitro dose response to DAla^{6}EA. Tissue slices (350 μm) of anterior pituitaries from female rabbits were incubated for 4 hours following the addition of DAla^{6}EA at increasing concentrations (10^{-11} M to 10^{-7}M). Results are expressed as amount of LH released per mg tissue weight. Values are the means ± SEMs of 3 observations. The effective dose giving half-maximal response (ED_{50}) was estimated as 0.6 nM by a computerized curve-fitting programme.


25
20
15
10
5
0
LH (ng/mg)
10⁻¹¹ 10⁻¹⁰ 10⁻⁹ 10⁻⁸ 10⁻⁷
DAla⁶EA (M)

CHAPTER FIVE

DISCUSSION

5.1 CHARACTERIZATION OF ^{125}I-DALA^{6}EA BINDING

A radioligand binding assay was established for the characterization of pituitary GnRH binding sites using DAla^{6}EA as labelled ligand. The results of the present study demonstrate the presence of specific ^{125}I-DAla^{6}EA binding sites in the anterior pituitary gland of the rabbit. ^{125}I-DAla^{6}EA binding was saturable (Figure 17), displaceable (Figure 15), reversible (Figure 21) correlated with increasing tissue concentration (Figure 11) and susceptible to physiological manipulation (Figures 16 and 18). In addition, competition curves revealed specificity of the ^{125}I-DAla^{6}EA binding sites (Figure 15). Thus, the ^{125}I-DAla^{6}EA radioligand assay fulfils many of the criteria for identification of specific pituitary GnRH receptors in the rabbit.

^{125}I-DAla^{6}EA Binding Constants: To initially characterize ^{125}I-DAla^{6}EA binding to rabbit pituitary membrane preparations, saturation experiments were conducted. Results indicated the presence of two binding sites at the concentration range tested (0.1-10 nM), a high affinity/low capacity site and a low affinity/high capacity site (Table 7). Displacement experiments also suggested that the rabbit anterior pituitary contains two classes of ^{125}I-DAla^{6}EA binding sites (Table 8). Similarly, GnRH binding to two sites has been reported in rat and bovine pituitary homogenates (Clayton et al, 1979). In contrast, GnRH analogs bind primarily to a single class of high affinity sites in the rat pituitary (Marian et al, 1981; Clayton et al, 1979). These same GnRH analogs were found to bind to two sites in the bovine (Clayton et al,

1979) and goldfish pituitary (Habibi et al, 1987). Thus, the present study supports previous data on the species differences evident in GnRH analog binding. The present findings of two ^{125}I-DAla^{6}EA binding sites are unique since previous studies using displacement data reported GnRH analog binding to a single class of binding sites in the rabbit pituitary (Limonta et al, 1986; Thorson et al, 1985). The significance of the presence of two binding sites in the rabbit anterior pituitary is unclear.

Binding constants for ^{125}I-DAla^{6}EA binding obtained from saturation experiments produced a high affinity binding site with a K_d of 0.39 ± 0.23 nM (B_{max}=32±10 fmol/mg protein) which was similar to the binding site reported by Limonta et al (1986) for the male rabbit pituitary (K_d=0.36 nM, B_{max}=110±8.9 fmol/mg protein) and Thorson et al (1985) for the female rabbit pituitary (K_d=0.14 nM, B_{max}=88 fmol/mg protein). The variability between studies in the B_{max} reported is probably a result of the type of pituitary tissue used (*i.e.* variation in age and sex of animals).

Tissue Specificity of ^{125}I-DAla^{6}EA Binding: In the present study high affinity ^{125}I-DAla^{6}EA binding sites were not detected in the ovary of the female rabbit. This is of interest since the high affinity of ^{125}I-DAla^{6}EA binding to the pituitary of the female rabbit was similar to both rat pituitary and ovarian tissues (K_d = 0.12 nM) previously reported by Pieper et al (1981). The absence of specific GnRH binding

sites in the rabbit ovary is in agreement with the data of Thorson et al (1985) and suggests that GnRH does not exert its effects by direct action on the rabbit ovary. Similarly, GnRH binding has not been observed in the gonads of hamsters (Theuring et al, 1987), mice (Wang et al, 1983), ewes, sows and cattle (Brown and Reeves, 1983). However, structural differences in peptides amongst species could account for the differences seen. It is possible that a binding site for an ovarian peptide that is not similar to DAla^{6}EA is present on the ovary of the rabbit and DAla^{6}EA may not be recognized by this binding site. The use of other GnRH analogs or the attempt to isolate a GnRH-like peptide from the ovary of the rabbit may clarify the presence or absence of specific ovarian GnRH binding sites. Nevertheless, the present study suggests that the rabbit is another species that does not contain GnRH binding sites in the ovary and GnRH-related peptides are not directly involved in the luteal function of the rabbit.

Although specific binding of ^{125}I-DAlaEA to rabbit hypothalamus was not found to be significant in the present study, there have been reports of specific hypothalamic (Heber et al, 1978) and hippocampal (Reubi et al, 1987) GnRH binding in the rat. The authors speculated that these binding sites suggest a role for GnRH in the CNS, possibly as a neurotransmitter or neuromodulator. GnRH binding sites in the hypothalamus may be involved in the ultra short-loop feedback effects of GnRH on the hypothalamus, regulating the secretion of GnRH (Heber et al, 1978). A physiological action of GnRH related to the hippocampus has not been

demonstrated and the relevance of these binding sites in the rat is uncertain. Further investigation of ^{125}I-DAla^{6}EA binding in the rabbit hypothalamus, with modification of the assay conditions and/or GnRH analog used, may clarify whether or not GnRH interacts with specific hypothalamic binding sites in the rabbit.

Interestingly, reports of GnRH analog binding in the rat liver, kidney (Aten et al, 1986) and adrenal cortical tissue (Pieper et al, 1981) have been documented. In contrast, specific ^{125}I-DAla^{6}EA binding was not significant in rabbit liver, adrenal or kidney (Table 6). However, because of the short plasma half-life of GnRH (2-4 minutes)(Handelsman and Swerdloff, 1986), it is unlikely that hypothalamic GnRH could reach these peripheral organs in significant amounts so the function of these GnRH binding sites in the rat remains to be elucidated. The possibility remains that another biologically cross-reactive molecule could utilize these binding sites on these organs. Alternatively, if GnRH were able to reach peripheral organs, these binding sites may be involved in the removal of GnRH from the peripheral circulation and subsequent degradation (Marshall et al, 1976).

Specificity of ^{125}I-DAla^{6}EA Binding: Validation of the GnRH analog DAla^{6}EA for the measurement of GnRH binding sites in rabbit pituitary membrane preparations was demonstrated by competitive inhibition studies. Unlabelled GnRH completely displaced ^{125}I-DAla^{6}EA from its binding site and the inhibition curves with GnRH and DAla^{6}EA were parallel (Figure 15). These results indicate that ^{125}I-

DAla^{6}EA binds to the same site as native GnRH in rabbit pituitary membrane preparations. The IC_{50} of native GnRH (10.8 μM) was much greater than that of DAla^{6}EA (1.36 nM). Previous studies in the rat pituitary have shown a greater affinity of GnRH analogs for pituitary binding sites when compared with native GnRH (Perrin et al, 1982; Clayton and Catt, 1980). In mammals, a higher rate of degradation of native GnRH compared to GnRH analogs occurs (Koch et al, 1977). The primary site of enzymatic cleavage of GnRH appears to be at the Gly^6-Leu^7 position with a secondary slower rate of inactivation at the C-terminal glycine amide residue (see Karten and Rivier, 1986). Substitution of a D-amino acid in position 6 protects these GnRH analog peptides from degradation (Karten and Rivier, 1986; Coy et al, 1976). Therefore, the lower IC_{50} of native GnRH when compared to DAla^{6}EA in the present study is probably due to enzymatic degradation of native GnRH.

Temperature Dependence of ^{125}I-DAla^{6}EA Binding: The ^{125}I-DAla^{6}EA radioligand binding assay was performed at 4°C for 60 minutes and under these incubation conditions no significant degradation of the tracer was evident (Table 8). However, at temperatures of 22°C, 30°C and 37°C specific ^{125}I-DAla^{6}EA binding was reduced (Figure 12). A time course of ^{125}I-DAla^{6}EA binding at 30°C showed an initial association rate which was slower than at 4°C followed by a progressive decrease in specific binding (Figure 13). These results may be due to degradation of

the tracer and/or instability of the pituitary membrane preparation at the higher temperature. In the rat pituitary, incubation at 23°C or 37°C resulted in a decrease in specific GnRH analog binding even in the presence of 1 mM bacitracin, suggesting that membrane instability may occur at these higher temperatures (Clayton and Catt, 1980). In the present study, the addition of bacitracin did not increase binding at 4°C suggesting that ligand instability is not a factor under these radioligand binding assay conditions.

Effect of Ions on ^{125}I-DAla^{6}EA Binding: Ca^{2+} has been established as a requirement for GnRH-stimulated LH release from the rat pituitary (see review by Conn et al, 1987). However, a permissive role for Ca^{2+} or other ions in the interaction between GnRH and its binding site has not been identified. Specific binding of GnRH analogs to rat pituitary membrane preparation was reduced in the presence of $CaCl_2$ (1mM), $MgCl_2$ (1mM), $MnCl_2$ (1mM), NaCl (10mM) (Keinan and Hazum, 1985; Marian and Conn, 1980), $LaCl_3$ (Marian and Conn, 1980) as well as other additional ions. In general, a correlation between cation charge and binding inhibition was revealed with trivalent > divalent > monovalent (Marian and Conn, 1980). Similarly, the present study demonstrated a reduction in ^{125}I-DAla^{6}EA binding to rabbit pituitary membrane preparations following the addition of $CaCl_2$, $MgCl_2$ and NaCl to the incubation medium (Figures 19A and 19B). These results suggest a non-specific action of ions in general on ^{125}I-DAla^{6}EA binding. Although not

investigated in the present study, the decreased GnRH binding to rat pituitary was not due to the addition of chloride and the decreased binding in the presence of Ca^{2+} was a result of a reduction in binding site affinity (Marian and Conn, 1980). These results suggest that any effect of Ca^{2+} or other ions on LH release from the pituitary must occur subsequent to ligand-binding site interaction.

Effect of Freezing on ^{125}I-DAla^{6}EA Binding: The results obtained from comparison of ^{125}I-DAla^{6}EA binding using fresh versus frozen tissues (quick frozen in liquid nitrogen, stored at -70°C) was surprising. Fresh pituitary preparations had significantly lower ^{125}I-DAla^{6}EA binding when compared to preparations from pituitaries frozen for 2 to 12 weeks (Figure 20). The affinity of ^{125}I-DAla^{6}EA binding did not change, although it should be noted that saturation data was fitted using a one-site model only due to limitations of the amount of tissue available. Thus, the present study cannot identify whether or not changes are occurring in the low or high affinity site, or both. The reason for the lower ^{125}I-DAla^{6}EA binding with fresh tissue is unclear. Perhaps the length of time taken to sacrifice the number of rabbits required (hours) compared to species such as rats (minutes) affected the binding results. Alternatively, freezing of tissue could produce an alteration in the pituitary membrane structure and this artifact was measured in the binding assay. It is also possible that significant amounts of endogenous GnRH were present in fresh pituitaries and were not removed during tissue preparation. In contrast to the present

results, Clayton and Catt (1981b) showed no difference in rat pituitary GnRH binding site content of freshly obtained glands and glands stored for 4 weeks at -70°C following rapid freezing on dry ice. The significance of these contradictory results in the rabbit are unclear.

Displacement of ^{125}I-DAla^{6}EA Binding: A high amount of NSB (as defined as that binding not displaced in the presence of an excess of unlabelled DAla^{6}EA) was found in ^{125}I-DAla^{6}EA binding to rabbit pituitary (40-45%). This NSB was higher than those levels reported by others in rats (Clayton et al, 1979) and using the present ^{125}I-DAla^{6}EA assay conditions (NSB 15-20% in female rats aged 44 days; data not shown). This level of NSB was similar to the 57% that was reported by Habibi et al (1987) using goldfish pituitaries. A peptide that cross reacts with DAla^{6}EA and attaches the ^{125}I-DAla^{6}EA may be present in the rabbit pituitary and could be responsible for the high level of NSB obtained. It is possible that other labelled GnRH analogs could have reduced the NSB in the present radioligand binding assay and would have been more suitable to the species used in the present study.

Functional Significance of ^{125}I-DAla^{6}EA Binding: Although radioligand binding studies provide useful information about the binding site, ligand and radioligand interactions, caution must be taken when interpreting the results. One major

consideration is that a binding site must be correlated to a physiological response for a binding site to be classified as a receptor. Recently, Habibi et al (1989) demonstrated a significant correlation between GnRH analog binding to the high affinity sites in the goldfish pituitary and gonadotropin release. A significant correlation was not found between biological activity and binding to the low affinity site or the affinity from a one site binding fit. Studies identifying two GnRH binding sites in rat and bovine pituitaries suggest that the GnRH-induced gonadotropin release is produced through the high affinity binding sites (Conn et al, 1987). The present study does not identify which one of the DAla^{6}EA binding sites in the rabbit pituitary is the functional receptor. However, the in vitro LH dose response curve to DAla^{6}EA administration (Figure 25) produced an ED_{50} of 0.6 nM which was similar to the K_d of the high affinity binding site (K_d = approximately 0.4 nM), and suggests that high affinity ^{125}I-DAla^{6}EA binding to the rabbit pituitary is likely correlated to LH release.

5.2 ONTOGENY OF PITUITARY ^{125}I-DALA^{6}EA BINDING IN THE FEMALE RABBIT

Sexual maturation in the rabbit is a complex process which is incompletely understood. Recently, an age-related prepubertal increase in gonadotropin secretion has been demonstrated in the female rabbit (YoungLai, 1986; deTurckheim et al, 1983). A discrete peak in LH and FSH was observed at 30-50 days after birth

(YoungLai, 1986; Wilkinson and YoungLai, 1986). Puberty normally occurs at approximately 100 days (YoungLai, 1986; deTurckheim et al, 1983). The role and mechanism of this changing pattern of gonadotropin during sexual maturation of the female rabbit is as yet unknown. The interaction between GnRH and its pituitary binding site is a possible site for modulation of gonadotropin secretion during sexual maturation. The present study investigated the possible role of GnRH binding in sexual maturation of the female rabbit by (a) single point ^{125}I-DAla^{6}EA binding analysis of rabbit pituitary membrane preparations of animals 25, 40, 50, 65, 75, 85 and 120 days of age and (b) saturation experiments to determine ^{125}I-DAla^{6}EA binding constants of animals aged 40, 75 and 120 days.

In addition, the relationship between pituitary ^{125}I-DAla^{6}EA binding and gonadotropin levels were investigated throughout maturation in the female rabbit. ^{125}I-DAla^{6}EA binding in individual pituitaries of rabbits aged 40 to 120 days was determined by single point analysis (Figure 23). The concentration of ^{125}I-DAla^{6}EA pituitary binding sites and serum LH varied throughout sexual maturation of the female rabbit (Table 11 and Figure 23). ^{125}I-DAla^{6}EA binding continued to increase during the prepubertal period (maximal binding at 85 days) followed by a slight but non significant decrease by 120 days. However, the change in ^{125}I-DAla^{6}EA binding was not correlated with gonadotropin levels, in fact ^{125}I-DAla^{6}EA binding continued to increase following 60 days of age, at a time when LH levels were declining. These results are in contrast to studies of the rat in which the concentration of pituitary

GnRH binding sites were found to be maximal in the prepubertal period, at a time when serum levels of gonadotropins were elevated (Chan et al, 1981). The lack of correlation between LH levels and ^{125}I-DAla^{6}EA binding in the present study suggests that gonadotropin secretion in the female rabbit is only partially explained by binding site number. An additional component, such as the post-receptor transduction or effector mechanism may form the cellular basis of gonadotropin release in the female rabbit.

Several reports have demonstrated a dissociation between pituitary GnRH binding sites and serum LH in other species. No change in GnRH binding site number or affinity was observed prior to the LH surge in cows (Leung et al, 1984) or hamsters (Adams and Spies, 1981). GnRH binding sites did not change during the estradiol-induced surge of gonadotropins in ewes (Wagner et al, 1979) although studies in the monkey show that there is an increase in GnRH binding sites before the LH surge (Adams et al, 1981). Furthermore, after gonadectomy of mice, GnRH binding sites decrease although LH and FSH are elevated (Naik et al, 1985) and gonadotrope responsiveness to GnRH is unchanged (Abbot et al, 1986). Thus, data demonstrating a dissociation between GnRH binding site numbers and serum LH is available to support the hypothesis that changes in post-receptor events may play a role in the circulating level of gonadotropins and the regulation of gonadotrope responsiveness.

The variation in ^{125}I-DAla^{6}EA binding to pituitaries of female rabbits of

varying ages appeared to be due to changes in the number of ^{125}I-DAla^{6}EA binding sites rather than in affinity as determined by saturation analysis (Table 12A). Saturation analysis of female rabbits aged 40, 75 and 120 days of age indicated the presence of two binding sites (Table 12B). The high affinity sites in all age groups were similar (K_d = 0.3 to 0.4 nM) while the low affinity site for 40 day rabbits was much lower (K_d=150 nM) than 75 and 120 days (K_d=30 nM). Although it may be tempting to speculate a change in the low affinity binding site with age, caution must be taken in interpreting the results due to the large variability in the lower affinity binding site data. The functional significance of these two sites, if any, is as yet unclear.

The only previous study to date on pituitary GnRH binding in female rabbits investigated ^{125}I-DAla^{6}EA binding in Dutch rabbits with a body weight of 2.2-2.7 kg (Thorson et al, 1985). Comparison of binding data of animals with similar body weights from the present study (75 days of age) showed a high affinity binding site with a K_d of 0.32 nM and B_{max} of 107 ± 69 fmol/mg protein which is similar to that reported by Thorson et al (1985)(K_d = 0.14 nM; B_{max} = 188 ± 36 fmol/mg protein).

Pituitary ^{125}I-DAla^{6}EA binding to rabbits 40, 75 and 120 days of age using saturation analysis (Table 12B) was not identical to that obtained by single point analysis (Figure 23). This discrepency may reflect (a) the limited information available using single point analysis or (b) the large variability in the data as a result of the small number of animals used per group (n=3). Alternatively, variation in the

time of year or day that animals were sacrificed may have affected the binding results. Ramirez et al (1986) reported that, in the female rabbit, annual variations in mean GnRH output occurs. Interestingly, GnRH output was low when rabbits were cannulated (push-pull apparatus) during the late spring and early summer and high during the late summer and early fall to spring months. The authors speculated that the GnRH apparatus may become more active during the fall and winter to prepare the hypothalamic-pituitary-ovarian axis of the rabbit for reproductive performance during the spring. Since GnRH has been shown to regulate its own binding site in the rat (Young et al, 1985; Detta et al, 1984), the changing pattern of GnRH secretion throughout the year in the female rabbit may be reflected in alterations in pituitary GnRH binding. In the present studies, animals were sacrificed during the months of November (single point analysis) and February (saturation analysis). In addition, treatment with melatonin has been shown to cause a reduction in GnRH binding site number (see Lang et al, 1990). A study done in the present laboratory demonstrated an increase in serum melatonin at 8 pm when compared to 8 am in the female rabbit (E.C. Todoroff et al, unpublished results). Although an attempt was made in the present study to standardize the time of animal sacrifice (7:30 am to 12:00 pm) it is not known if changing patterns of melatonin or GnRH occurring daily or seasonally in the female rabbit influence pituitary ^{125}I-DAla^{6}EA binding.

5.3 DOWN REGULATION OF ^{125}I-DALA^{6}EA BINDING

To establish if GnRH has a role in the regulation of its own binding site, female rabbits were injected with 100 ng DAla^{6}EA per kg body weight every 8 hours for 4 days. DAla^{6}EA administration decreased ^{125}I-DAla^{6}EA binding site number by 80 percent when compared to control animals using single point analysis (Figure 23). These results are consistent with the hypothesis that GnRH binding site numbers are regulated by endogenous GnRH secretion. Binding site affinity, as determined by saturation analysis, did not change (K_d=3-4 nM)(Table 10). However, it should be noted that saturation data was fitted to one (overall) binding site only due to limited availability of tissue. Nevertheless, saturation results support the idea that endogenous GnRH regulates pituitary GnRH binding site number in the female rabbit. These data are in agreement with previous studies in the rat demonstrating down regulation of GnRH binding sites following long term exposure to GnRH or GnRH analogs (McArdle et al, 1987a).

Desensitization of the pituitary gonadotropes, as indicated by a decrease in the release of LH in response to a subsequent injection of GnRH, occurs after chronic exposure to GnRH. Evidence that down regulation of the GnRH binding site is responsible for the reduction in the ability of the pituitary to respond to GnRH has been demonstrated in the rat (Nett et al, 1981). However, some studies do not show a correlation between gonadotrope responsiveness and GnRH binding site number following continuous GnRH stimulation (Adams et al, 1986). Thus, desensitization

may involve a disruption of the post-receptor transduction or effector mechanism(s). Interestingly, in the present study, the serum LH of control animals and DAla^{6}EA treated animals (down regulated; 80 percent decrease in ^{125}I-DAla^{6}EA binding) were similar (LH approximately 0.5 ng/ml). This could suggest that LH secretion in the female rabbit is not directly correlated with the number of GnRH binding sites. Alternatively, the sensitivity of the RIA may not be sufficient to demonstrate any noticeable change in LH following long-term DAla^{6}EA injection. Whether or not the down regulation of ^{125}I-DAla^{6}EA binding sites in the female rabbit results in gonadotrope desensitization is not known from the present study and requires further investigation.

5.4 IN VIVO RESPONSE TO DALA^{6}EA

Sexual maturation in mammals is characterized by altered pituitary responsiveness to GnRH. In the rat, the pituitary response to exogenous GnRH increases with age and is maximal in the prepubertal period followed by a decline prior to first ovulation (Ojeda et al, 1977; Debeljuk et al, 1972). Peak responses to GnRH of male and female rats is correlated with high concentrations of pituitary GnRH binding site number (Duncan et al, 1983; Dalkin et al, 1981). This temporal relationship between GnRH binding site number and response to GnRH has led to the hypothesis that changes in GnRH binding is one mechanism by which pituitary responsiveness is altered during sexual development (Dalkin et al, 1981). To

determine if responsiveness to GnRH changes during sexual maturation in the female rabbit, animals aged 40, 75 and 120 days were injected with DAla^{6}EA (10ng, 100ng and 1μg per kg body weight). Serum LH was analyzed at time periods 0, 15, 30, 45 and 120 minutes post DAla^{6}EA injection.

All age groups of rabbits (40, 75 and 120 days) responded in a dose-dependent manner to DAla^{6}EA injection. Thus, the hypothalamic-pituitary axis of the female rabbit is already functional at the age of 40 days. The high and sustained LH response to 100 ng and 1 μg DAla^{6}EA at 120 minutes for animals aged 120 days would be expected to cause ovulation. This response at 120 minutes was significantly higher when compared to animals 40 and 75 days of age and suggests that at 120 days of age the female rabbit pituitary responds differently to GnRH than at earlier ages.

The net peak response to DAla^{6}EA (as measured by the change in LH at 30 minutes) was not significantly different amongst the three age groups of female rabbits (Table 14). Similarly, YoungLai et al (1986) found no net change in LH response to the administration of 1 μg/kg Gonadorelin Hydrochloride (Factrel) in female rabbits aged 26-100 days. These results are in contrast to the female rat which responds to GnRH with less LH secretion as the animal approaches sexual maturation (Debeljuk et al, 1972). However, it is also possible that the interval of blood sampling (15 minutes) in both studies in the female rabbit was too long to detect any significant initial change in LH release. In addition, the present study is

limited to magnitude of LH response alone. Changes in the dynamics of LH release (rate of rise or decay of LH) or the morphology of pituitary gonadotropes may occur during sexual maturation.

Of interest is the lack of LH response to DAla^{6}EA at 75 days at the dosage 10 ng/kg. In the rat, a decline in sensitivity of the pituitary to GnRH is believed to be the first step in the initiation of sexual maturation (Naish et al, 1986; Wilkinson and Moger, 1981). Using an in vitro system, isolated rat pituitaries were more responsive to GnRH between 20 and 30 days of age with puberty occurring at approximately 35 days (Wilkinson and Moger, 1981). Similarly, the GnRH-stimulated LH secretion of superfused rat pituitaries was greater at 10-20 days of age when compared to animals 30 days of age (Naish et al, 1986). Thus, the minimal LH response at 15 minutes following 10 ng/kg DAla^{6}EA administration to Day 75 rabbits when compared to 40 days and 120 days of age may suggest a change in responsiveness to GnRH in the female rabbit that is occurring prior to sexual maturation. The other two high doses of DAla^{6}EA administered (100 ng/kg and 1 μg/kg) may have masked any decrease in LH response present.

In the female rabbit, the magnitude of the increase in pituitary ^{125}I-DAla^{6}EA binding site number measured between 40 day and 75 days of age was in the order of 3-4 times. However, the net change in LH in response to DAla^{6}EA was not significantly different between rabbits aged 40 days and 75 days. If this lack of increase in LH is taken as an index of the measure of pituitary responsiveness, then

pituitary responsiveness in the rabbit is not related to GnRH binding site number at these ages. Previous studies in other species have shown that LH responsiveness is not functionally linked to GnRH binding site number. For example, the magnitude and direction of changes in GnRH binding sites following ovariectomy did not account for the changes in pituitary responsiveness to GnRH in the ewe (Clarke et al, 1988) or rat (Pieper et al, 1984). However, in monkeys the alterations in GnRH responsiveness are of the same magnitude as changes in GnRH binding site number (Adams et al, 1981). The present results in the female rabbit indicate that additional factors may be involved in the LH response to GnRH. The investigation of post receptor events of GnRH action, as discussed in the following section, may provide more insight into the relationship between binding site number and pituitary responsiveness.

5.5 FUTURE CONSIDERATIONS

1. What role(s) do gonadal steroids (estradiol, progesterone) have in the regulation of the GnRH binding site of the female rabbit?

The changes in serum gonadotropins that occur following ovariectomy have been well documented (see Clayton and Catt, 1981a). In the rat, castration results in an increase in gonadotropins which can be prevented by steroid replacement (Frager et al, 1985). The precise mechanisms that produce the gonadotropin rise are not

completely understood. Increased levels of GnRH in pituitary stalk blood has been reported in ovariectomized rhesus monkeys and may be responsible for gonadotropin elevation (Neill et al, 1977). Since GnRH has been shown to regulate the number of pituitary binding sites in the pituitary gland, the increase in GnRH secretion following ovariectomy could result in an increase in GnRH binding site number. In the rat, GnRH binding site number has been shown to increase following castration without a change in binding site affinity (Frager et al, 1985). In contrast to the rat, the concentration of pituitary GnRH binding sites were significantly reduced after castration in mice (Clayton et al, 1985) and no change was observed in hamsters (Pieper et al, 1982). Thus, species differences exist in the modulation of GnRH binding sites by gonadal steroids. In male rabbits, pituitary GnRH binding sites significantly increased after castration and the administration of testosterone suppressed the castration-induced increase (Limonta et al, 1986). These results suggest a similarity between male rabbits and rats in the regulation of the hypothalamic pituitary axis (Limonta et al, 1986). However, the hormonal regulation of pituitary GnRH binding sites in the female rabbit remains to be determined.

2. What is the second messenger(s) involved in GnRH action in the female rabbit pituitary ?

The cellular events following GnRH receptor activation include signal transduction and the generation of intracellular effector molecules. Studies in the rat

have demonstrated that the molecular mechanism of GnRH and LH release is a complex process (see Naor, 1990a). It appears that two pathways are involved in GnRH action which results in a biphasic stimulation of LH release (Smith et al, 1989). One pathway is initiated within seconds of GnRH-receptor activation and results in an increase in intracellular Ca^{2+} (Chang et al, 1986; Clapper and Conn, 1985). This initial mobilization of Ca^{2+} is believed to be due to IP_3 which accumulates within seconds of GnRH receptor stimulation (Naor et al, 1986) due to activation of the membrane bound enzyme phospholipase C (PLC)(Berridge, 1984). An initial rapid phase of LH release from the pituitary occurs. A second pathway is also Ca^{2+} dependent but the source of Ca^{2+} is extracellular entering the gonadotrope through voltage-dependent Ca^{2+} channels (Stojilkovic et al, 1988; Chang et al, 1986). This increase in extracellular calcium has been shown to produce the sustained LH release (Hansen et al, 1987). The second product of PLC activation is diacylglycerol which activates the enzyme protein kinase C. Protein kinase C has been implicated in GnRH action since synthetic diacylglycerols (Harris et al, 1985) and phorbol esters (McArdle et al, 1988; Harris et al, 1985) stimulate LH release. Investigation of these post-receptor events is critical to the understanding of the molecular mechanism of GnRH action in the female rabbit pituitary.

3. Does GTP alter GnRH binding in the female rabbit pituitary, *i.e.* is there a G-protein involved in GnRH action ?

Guanine nucleotide binding proteins (G-proteins) are a family of proteins located on the cytoplasmic surface of cell membranes. For a variety of ligands, G-proteins act as a transducer to couple the receptor with the effector molecule. G-proteins consist of α, ß and γ-subunits. The α-subunit varies amongst G-proteins but the ß and γ-subunits appear to be similar. In the unstimulated state, the α-subunit is bound to GDP. Following binding of a ligand to its receptor, GDP is replaced by GTP and the complex dissociates into α and ßγ-subunits. The α-GTP then modulates the activity of the effector molecule (e.g. adenylate cyclase, phospholipase C). GTP is converted to GDP by intrinsic GTPase activity which results in reassociation of the three subunits and deactivation of the process (see Birnbaumer et al, 1991).

Only recently has the possible involvement of a G-protein coupling the GnRH binding site to phosphoinositol production and LH release been investigated. Analogs of GTP reduce the binding of GnRH agonists in bovine and rat pituitary membranes (Perrin et al, 1989; Limor et al, 1989) which is consistent with an association of the GnRH binding site and a G-protein. Andrews et al (1986) demonstrated that the addition of GTP or GTP analog stimulated a time- and dose dependent increase in inositol phosphate accumulation and LH release in permeabilized rat pituitary cells. Treatment of pituitary cells with sodium fluoride, an exogenous activator of G-proteins, resulted in an accumulation of inositol phosphates and LH release (Waters et al, 1990). Since the responses to GTP or sodium fluoride were unaffected by pertussis toxin and cholera toxin, the G-protein mediating GnRH action appears to

be different from the G-protein that regulates adenylate cyclase (Andrew et al, 1986; Waters et al, 1990). Further studies are necessary to characterize the G-protein coupled to the GnRH binding site and establish their role in signal transduction.

4. What molecular model(s) could account for the observed complex binding phenomena?

^{125}I-DAla^{6}EA binding to the rabbit pituitary membrane preparation resulted in a curvilinear downward Scatchard plot in a number of experiments (see Figure 26B). These complex binding data could be explained by a number of molecular interactions, including:

(a) negative co-operativity of binding sites in which the affinity of the binding site population decreases as fractional occupancy increases,

(b) multiple independent populations of binding sites which have separate and unchanging affinities, or

(c) multiple affinity states of the binding site for the ligand such as hormone-receptor interactions involving the formation of a ternary complex (H+R ↔ HR+T ↔ HRT).

The present study cannot distinguish between the above possibilites. Several independent studies are necessary to identify a model which could explain the observed heterogenous interaction between ^{125}I-DAla^{6}EA and its pituitary binding site in the female rabbit.

One method of analyzing complex curves is the Hill (1913) transformation of saturation binding data. A Hill plot can be obtained by plotting the log of radioligand concentration (log[L]) against the log of [B/B_{max} - B], where B is the amount of radioligand bound and B_{max} is the maximum number of binding sites. The slope of the line is denoted as the Hill coefficient. Lack of co-operativity is suggested by a slope of 1.0. The value of a Hill coefficient less than 1.0 indicates negatively co-operative interactions or the possibility of radioligand binding to a heterogenous population of binding sites. In addition, the presence or absence of co-operativity of ^{125}I-DAla^{6}EA may be determined by dissociation experiments. The dissociation rate is determined by (a) diluting the incubation medium with an excess (~50 fold) of incubation buffer and (b) diluting the incubation medium in the presence of an excess (10^{-6}M) of unlabelled DAla^{6}EA. If both the rates and extent of dissociation are the same, the multiple binding site model would be favoured over the negative co-operativity model.

A number of receptor systems have demonstrated the existence of multiple binding site affinity states for the agonist. These varying affinity states may involve the formation of a ternary complex of hormone action (HRT) where T represents a G-protein in many cases (Rodbell, 1985). In systems whose receptors are linked to G-proteins, the presence of GTP will result in the receptor being expressed in its low affinity form. Since recent evidence suggests that a G-protein is involved in the action of GnRH at the pituitary (Waters et al, 1990), this ternary model may account for

the complex curves observed in ^{125}I-DAla^{6}EA binding to rabbit pituitary membranes. Experiments using an excess of GTP or guanine nucleotide analogs (e.g. Gpp[NH]p) would result in all of the binding sites being converted to a low affinity state. Positive results from these GTP experiments may suggest that the curvilinear Scatchard plots of ^{125}I-DAla^{6}EA binding to rabbit pituitary membrane preparations represent two affinity states of the GnRH binding site. Nevertheless, it is necessary to combine a number of independent experimental approaches to determine the appropriate model to describe the complex binding data observed. Although several alternatives are available for interpretation of binding data, the present study utilized the two independent site model for analysis of ^{125}I-DAla^{6}EA binding to the female rabbit pituitary.

5.5 CONCLUSIONS

The major contributions of the present study are the following:

1. The pituitary GnRH binding site of the female rabbit was characterized using the radioligand binding assay with ^{125}I-DAla^{6}EA as labelled ligand. Despite some as yet unresolved characteristics of the binding, the ^{125}I-DAla^{6}EA radioligand assay promises to be useful for the future investigation of the role of the pituitary GnRH binding site in the female rabbit.

2. ^{125}I-DAla^{6}EA binding indicated the presence of two binding sites in the rabbit pituitary, a high affinity/low capacity site and a low affinity/high capacity site. The functional significance of these two sites remains to be elucidated.

3. Significant ^{125}I-DAla^{6}EA binding was not present in the rabbit ovary suggesting that GnRH or GnRH-related peptides are not directly involved in the control of the luteal function of the rabbit.

4. ^{125}I-DAla^{6}EA binding in the female rabbit pituitary was not correlated with serum LH throughout sexual maturation (40 to 120 days of age). This indicates that post-receptor events may play a role in gonadotropin secretion.

5. The net peak serum LH response to DAla^{6}EA did not change in female animals with age under the conditions of the present study, suggesting that a decline in pituitary responsiveness to GnRH is not associated with sexual maturation in the female rabbit.

6. A simple receptor-ligand interaction model cannot describe GnRH binding to the rabbit pituitary. Future studies investigating the effector and/or transducer molecules involved in GnRH binding to the rabbit pituitary are necessary for understanding the complex action of GnRH.

APPENDICES

APPENDIX A

Lowry Protein Determination

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and R.J. Randall
J Biol Chem 193: 265-275, 1951.

Solution A
20 g Na_2CO_3/litre in 0.1N NaOH or 23.4 g $Na_2CO_3.H_2O$
i.e. 4 g NaOH in 1 litre volumetic flask, dissolve
add 20 g Na_2CO_3 and make up to 1 litre with distilled water

Solution B
0.5 g $CuSO_4.5H_2O$ in 1 % potassium sodium tartrate (100 ml)
dissolve 1 g potassium sodium tartrate in 100 ml dH_2O
add 0.5 g $CuSO_4.5H_2O$
add 1 pellet NaOH to clear solution and turn dark blue

Solution C
50 ml solution A + 1 ml solution B (make fresh)

Protein Stock
1 mg BSA in 1 ml dH_2O (make fresh)

Procedure
1. blanks (2) 250 µl buffer (any buffer without gel)
2. samples and standards made up to total volume of 250 µl with buffer, duplicates
3. add 2.5 ml solution C to all tubes
4. vortex. Let stand 15 min at room temperature
5. add 0.25 ml Folins (2 N Phenol)
   1 ml 2 N Phenol + 1 ml distilled H_2O
6. vortex. Let stand 30 minutes at room temperature
7. read spectrometer at a wavelength of 560 nm

Standards
10-125 µg protein (10-125 µl stock)

Analysis of Results
linear regression analysis
reading X dilution /amount tissue used = µg protein/µl

APPENDIX B
Iodination of DAla^{6}-des Gly^{10}-GnRH ethylamide

Clayton, R.N. Methods in Enzymology. 103: 32-48, 1983.

1. Dilute 5 μg of GnRH analog in 10 μl of 0.1 M phosphate buffer (pH 7.5). Store in aliquots in freezer at -70°C.

2. To 5 μg of GnRH analog:
   add 10 μl of 0.1 M phosphate buffer (pH 7.5)
   add 1.5 mCi ^{125}I-Na in 10 μl
   add 10 μl of chloramine-T (5μg/10μl) in 0.1 M PO_4 buffer.
   Chloramine-T is prepared immediately before use.

3. Mix reagents by flicking with finger for 5 minutes.

4. Add 400 μl of 0.1 M PO_4 buffer.

5. Using a pipette coated with 1% PBS/BSA, transfer contents of vial to a glass tube containing 200 μg Dowex presoaked in 300 μl 0.1 M PO_4 buffer.

6. Mix for 30 seconds.

7. Centrifuge at 700 x g for 5 minutes.

8. Carefully aspirate supernatant and apply to Sephadex G-25 fine column (1 x 55 cm).
   Elute with 0.1 N acetic acid containing 0.25% BSA (pH~4.5).
   Collect 1.5 ml fractions.
   Peak routinely appears at tubes 20-26.

9. All procedures are carried out at room temperature.

APPENDIX C
Hematoxylin and Eosin Staining

Reagents

acid water	-	1.5 ml HCl/150 ml dH_20
ammonia water	-	6 drops NH_4OH/150 ml dH_20
1% ethyl eosin	-	10 g eosin Y dissolved in 50 ml dH_2O add 940 ml of 95% ETOH
hematoxylin	-	use Ehrlich's Original Formula

Tissue Fixation and Blocking

1. place tissue into Davidson's Fixative (24-36 hrs)
2. following day 70% ETOH - 60 min
3. 80% ETOH - 60 min
4. 90% ETOH - 60 min
5. 95% ETOH - 60 min
6. 95% ETOH - α-terpineol (1:1), 60 min
7. α-terpineol - 3 changes, 60 min each
8. melted paraffin: α-terpineol - overnight
9. following day paraffin wax - 3 changes, 60 min each
10. block tissue with paraffin - stainless steel base containing plastic embedding ring

Sectioning

Paraffin blocks cut on microtome (10μm) and placed on albuminized slides. Slides allowed to dry overnight on warming plate.

Staining

1. xylene - 3 changes, 3 min each
2. 100% ETOH - 2 changes, 2 min each
3. 95% ETOH - 2 changes, 2 min each
4. 80% ETOH - 1 change, 1 min
5. 70% ETOH - 1 change, 2 min
6. 50% ETOH - 1 change, 2 min
7. distilled water - 1 change, 2 min (change H_2O each time)
8. hematoxylin - 10-20 min (use 12 min)
9. tap H_2O - rinse in running H_2O thoroughly

10. acid H_2O - 4 to 6 dips, tissue will turn red
11. tap H_2O - rinse in running H_2O
12. ammonia H_2O - 4 to 6 dips, tissue will turn red
13. running tap H_2O - 5 to 10 min
14. 50% ETOH - 2 min
15. 70% ETOH - 2 min
16. 80% ETOH - 2 min
17. eosin - 1 dip, 30 seconds
18. 95% ETOH - 3 changes, 2 min each
19. 100% ETOH - 3 changes, 2 min each
20. xylene - 3 changes, 3 min each

APPENDIX D
Iodination of Rabbit Luteinizing Hormone

1. Dilute 2 μg of LH antigen (LER-1056-C2) in 10 μl of 0.1 M phosphate buffer (pH 7.5).
   Store in aliquots in freezer at -70°C.

2. To 2 μg of LH antigen:
   add 10 μl of 0.1 M phosphate buffer (pH 7.5)
   add 0.5 mCi ^{125}I-Na in 10 μl
   add 10 μl of chloramine-T (25mg/5ml) in 0.1 M PO_4 buffer.
   Chloramine-T is prepared immediately before use.

3. Mix reagents by flicking with finger for 45-60 seconds.

4. Add 20 μl of sodium metabisulphite ($Na_2S_2O_5$) in 0.1 M PO_4 buffer(25mg/8ml).

5. Mix reagents for 30 seconds.

6. Carefully aspirate mixture and apply to a Sephadex G-75 column (1 x 30 cm).
   Elute with 0.01 M PBS (pH 7.8).
   Collect 10 drops per tube.
   Peak routinely appears at tubes 3-6.

7. All procedures are carried out at room temperature.

APPENDIX E
Radioimmunoassay of Rabbit Luteinizing Hormone

Standards

Rabbit LH - WP 360A from Dr. A.F. Parlow
1.5 ng = 40 pg purified rabbit LH

A. 10μg/ml = 1000 ng/100μl make dilutions in 1% BSA/PBS
B. 0.5 ml A + 4.5 ml 1% BSA/PBS = 100 ng = 2.66 ng LH/100μl
C. 2 ml B + 2 ml 1% BSA/PBS = 50 ng = 1.33 pg LH/100μl
D. 2 ml C + 2 ml 1% BSA/PBS = 25 ng = 665 pg LH/100μl
E. 2 ml D + 2 ml 1% BSA/PBS = 12.5 ng = 333 pg LH/100μl
F. 2 ml E + 2 ml 1% BSA/PBS = 6.25 ng = 167 pg LH/100μl
G. 2 ml F + 2 ml 1% BSA/PBS = 3.125 ng = 84 pg LH/100μl
H. 1 ml G + 1 ml 1% BSA/PBS = 1.563 ng = 42 pg LH/100ul

First Antibody

Guinea pig anti-rabbit 6F GPāLH (from Dr. R. Scaramuzzi)
Have 1: 1000 dilution in 2% NGPS/EDTA/PBS
1:30,000 dilution is good for 30-50% binding in zero tubes.

Second Antibody

Goat anti-guinea pig gamma globulin. Dilute 1:10 in PBS.

Unknown Sera

Use 25 μl to 100 μl. Make up the difference with 1% BSA/PBS.

Protocol

Tube	1,2,3	100 μl 1% BSA/PBS
	4,5,6	100 μl 1% BSA/PBS
	7,8	100 μl 42 pg LH
	9,10	100 μl 84 pg LH
	11,12	100 μl 167 pg LH
	13,14	100 μl 333 pg LH
	15,16	100 μl 667 pg LH
	17,18	100 μl 1330 pg LH

19,20	100 µl 2660 pg LH
21,22	quality control low
23,24	quality control medium
25,26	quality control high
27-end	unknowns

Day 1: Add 50 µl NGPS/EDTA/PBS to tubes 1-3.
Add 50 µl 1st Antibody to tubes 4,5,6-end.
Mix. Keep covered at 4°C.

Day 2: Add 50 µl (10,000 cpm) tracer to all tubes plus 3 extra empty tubes for total activity.

Day 4: Add 100 µl 2nd Antibody to all tubes except 3 tracer tubes. Mix. Return to 4°C.

Day 5: Count tracer tubes. Centrifuge other tubes at 4°C (30-45 min at 600 x g). Aspirate supernatant. Count precipitate.

APPENDIX F
Buffers Routinely Used

Tris-HCl (50 mM, pH 7.4)

Dissolve 23.23 g of tris(hydroxymethyl) aminomethane in 3.5 L ddH_20. Add 30 ml 6M HCl. Bring up to 4 litres with ddH_2O. Place in fridge for 2-3 hours. Adjust to pH 7.4 with Tris Base or HCl. Store at 4°C.

Phosphate Stock Solutions

(a) Dissolve Na_2HPO_4 (28.4 g) in hot water. When cold, dilute to 500 ml with dH_2O. Store at 4°C. Dissolve in warm water when ready to use if crystals form.
(b) Dissolve KH_2PO_4 (13.61 g) in water to 100 ml. Store at 4°C.

Phosphate buffer (0.4 M, pH 7.5)

Dilute 10 ml of stock solution (b) with distilled water to a final volume of 25 ml (0.4 M). Add ~15-16 ml of this to ~70 ml stock solution (a) until pH 7.5 is reached. Store at 4°C.

PBS (0.01 M, pH 7.8)

Add 0.88 g NaCl to 5-10 ml of stock solution (b). Add dH_2O to 100 ml. Dissolve 8.8 g NaCl in 25 ml stock solution (a). Add distilled water to 1000 ml. Then slowly, while stirring, add the first solution to the second until pH 7.8 is reached. Add sodium azide to 0.01%. Store at 4°C.

2% Normal Guinea Pig Serum/PBS/EDTA

Dissolve 1.86 g $Na_2EDTA.2H_2O$ in 100 ml PBS. Measure 49 ml and add 1 ml normal guinea pig serum.

APPENDIX G
Calculation of Specific Activity

Specific activity of ^{125}I-labelled DAla^{6}EA was estimated using adult (45 days of age) female rat pituitary homogenates. Simultaneous fitting of displacement and saturation curves were carried out. Specific activity was calculated by comparing the cpm that produces 50% maximal specific binding with the quantity of unlabelled DAla^{6}EA that displaced 50% of the label.

Example

250000 cpm ^{125}I-DAla^{6}EA = 90 pg DAla^{6}EA
dpm = cpm x efficiency of counter (85%)
250000 cpm x 100/85 = 294118 dpm
294118 dpm = 90 pg
1 μCi = 2.22 x 10^6 dpm
0.132 μCi = 90 pg
0.001472 μCi = 1 pg
1.472 μCi = 1 ng
1472 μCi = 1 μg
Specific Activity = 1472 μCi/μg

Concentration of Radioactive Ligand

$$\frac{\text{mCi/ml x 1000}}{\text{specific activity (Ci/mmol) x 2.22}} = \mu M$$

Concentration of Radioactive Ligand from Dpms

$$\frac{\text{dpm}}{\text{specific activity (Ci/mmol) x 2.22}} = \text{fmoles}$$

$$\frac{\text{dpm/ml}}{\text{specific activity (Ci/mmol) x 2.22}} = \text{pM}$$

1 Ci = 2.22 x 10^{12} dpm

APPENDIX H

TABLE H.1
Analysis of Variance Table for Pituitary Weights of Female Rabbits (25 to 120 days of age)

Source	DF	SS	MS	F	P
Age (A)	6	49.00	8.18	33.17	0.00
Rep (B)	5	0.42	0.08	0.34	0.88
A*B	15	3.69	0.24		
Total	26	53.19			

TABLE H.2
Scheffés Pairwise Comparison of Pituitary Weights by Age

Age (days)	Mean (mg)	Homogeneous Groups
120	4.87	I
75	3.30	I
85	3.09	I
50	2.77	I I
65	2.48	I I
40	1.73	I I
25	1.23	I

There are 4 groups in which the means are not significantly different from one another.
Critical F Value 2.79 Rejection Level 0.05
Critical Value for Comparison 1.17
Standard Error for Comparison 0.28
Error Term Used: age*rep, 15 df

TABLE H.3
Analysis of Variance Table for Body Weights of Female Rabbits (25 to 120 days of age)

Source	DF	SS	MS	F	P
Age (A)	6	4.4E+07	7.4E+06	190	0.00
Rep (B)	5	2.6E+05	5.2E+04	1.36	0.28
A*B	18	6.9E+05	3.8E+04		
Total	29	4.5E+07			

TABLE H.4
Scheffés Pairwise Comparison of Body Weights by Age

Age (days)	Mean (g)	Homogeneous Groups
120	3808	I
75	2563	I
85	2911	I
50	1613	I
65	2148	I
40	1168	I
25	475	I

There are 6 groups in which the means are not significantly different from one another.
Critical F Value 4.67 Rejection Level 0.05
Critical Value for Comparison 374.97
Standard Error for Comparison 113.46
Error Term Used: age*rep, 18 df

TABLE H.5
Analysis of Variance Table for Serum LH of Female Rabbits (25 to 120 days of age)

Source	DF	SS	MS	F	P
Age (A)	5	24.38	4.87	4.93	0.00
Rep (B)	8	9.00	1.12	1.14	0.38
A*B	24	23.75	0.98		
Total	37	57.15			

TABLE H.6
Scheffés Pairwise Comparison of Serum LH by Age

Age (days)	Mean (ng/ml)	Homogeneous Groups
50	2.28	I
40	1.27	I
85	0.53	I
75	0.51	I
25	0.43	I
120	0.59	I

There are 2 groups in which the means are not significantly different from one another.
Critical F Value 2.62 Rejection Level 0.05
Critical Value for Comparison 1.69
Standard Error for Comparison 0.47
Error Term Used: age*rep, 24 df

APPENDIX I
Raw Binding Data
120 Day Female Rabbit

Total (dpm)	NSB (dpm)	Specific (dpm)
2048	280	1768
3061	491	2570
3942	735	3207
6387	745	5642
5410	957	4453
6975	913	6062
8801	1876	6925
11414	2163	9251
10030	1822	8208
14190	2612	11578
12345	2921	9424
18828	3841	14987
29992	7110	22882
38683	17789	26894

Bound ligand (specific binding, dpm) and total ligand added (dpm) were converted to pM values (see Appendix G). The appropriate values were entered into the BDATA programme. Analysis was performed by fitting the experimental data to one or two independent binding sites. A non-linear least squares parametric fit of the experimental Scatchard curve estimated the apparent dissociation constant (K) and number of binding sites (N). Results from the above representative experiment are shown on the following pages.

RESULTS OF PARAMETRIC FIT

The fitted parameters are :
Set # 1
K = 4572.085
N = 405.1895

K_d= 4.57 nM

$$B_{max}= \frac{408 \times 400ul}{57.7\ ug} = 2908 \text{ fmol/mg protein}$$

Residuals = 1.009021E-03
Degrees of Freedom = 11

Bexp	Bcalc	B/Fexp	B/Fcalc	Residuals
36.0000	30.3354	0.0973	0.0820	0.0153
52.0000	57.8182	0.0683	0.0760	-0.0076
65.0000	81.7159	0.0563	0.0707	-0.0145
115.0000	100.8467	0.0759	0.0666	0.0093
123.0000	139.7449	0.0511	0.0581	-0.0070
141.0000	157.2254	0.0486	0.0542	-0.0056
188.0000	171.9895	0.0558	0.0510	0.0047
192.0000	255.5492	0.0246	0.0327	-0.0081
198.0000	208.6123	0.0408	0.0430	-0.0022
235.0000	225.9745	0.0408	0.0392	0.0016
305.0000	276.2448	0.0311	0.0282	0.0029
465.0000	308.9880	0.0317	0.0210	0.0106
546.0000	328.7199	0.0278	0.0167	0.0111

B (pM)	X (pM)	XT (pM)	LogX
36	370	406	2.568202
52	761	813	2.881385
65	1155	1220	3.062582
115	1515	1630	3.180413
123	2407	2530	3.381476
141	2899	3040	3.462248
188	3372	3560	3.527888
192	7808	8000	3.89254
198	4852	5050	3.685921
235	5765	6000	3.760799
305	9795	10100	3.991005
465	14685	15150	4.166874
546	19654	20200	4.293451

RESULTS OF PARAMETRIC FIT

The fitted parameters are :

Set # 1

K = 347.0776 K_{d1} = 0.35 nM

N = 38.02567 B_{max1} = 263 fmol/mg protein

Set # 2

K = 23525.29 K_{d2} = 23.5 nM

N = 993.2357 B_{max2} = 6883 fmol/mg protein

Residuals = 6.318311E-04

Degrees of Freedom = 9

Bexp	Bcalc	B/Fexp	B/Fcalc	Residuals
36.0000	35.0001	0.0973	0.0946	0.0027
52.0000	57.2377	0.0683	0.0752	-0.0069
65.0000	75.7212	0.0563	0.0656	-0.0093
115.0000	91.0312	0.0759	0.0601	0.0158
123.0000	125.4243	0.0511	0.0521	-0.0010
141.0000	142.9274	0.0486	0.0493	-0.0007
188.0000	158.9948	0.0558	0.0472	0.0086
192.0000	283.9135	0.0246	0.0364	-0.0118
198.0000	205.3124	0.0408	0.0423	-0.0015
235.0000	231.3579	0.0408	0.0401	0.0006
305.0000	328.7010	0.0311	0.0336	-0.0024
465.0000	418.8686	0.0317	0.0285	0.0031
546.0000	489.4588	0.0278	0.0249	0.0029

To test if the curve fitting to a two site model was statistically significant, an F ratio was calculated from the equation:

$$F = \frac{\dfrac{(SSR_1 - SSR_2)}{df_1 - df_2}}{\dfrac{SSR_2}{df_2}} = \frac{\dfrac{10.1 - 6.31}{11 - 9}}{\dfrac{6.31}{9}} = 2.70$$

where SSR_1 is the sum of squared residuals for the first model and SSR_2 the sum of squared residuals for the model with an additional set of binding sites. df_1 and df_2 are the degrees of freedom of the first and second model respectively. The calculated F ratio was compared to the tabulated ratio for the F statistics with $(df_1 - df_2)$ and df_2 degrees of freedom.

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